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APPLICATIONS OF PRIMARY CELL CULTURES IN THE STUDY OF ANIMAL VIRUSES†

I. The Isolation and Characterization of Bovine and Avian Enteric Viruses¶

Tissue culture has been used for the study of animal viruses since 1906 when Aldershoff and Boers first grew vaccinia in rabbit tissue separate from the whole host.[3] Other limited successes were obtained in the years following, but extensive use of tissue culture in animal virology awaited the studies of Enders, Weller, and Robbins[9] on a human agent, poliovirus. Since then, the use of tissue culture techniques has led to a revolution of methodology in the whole field of animal virus research, entirely comparable to that in the human virus field. Methods of cell dispersal developed by Dulbecco and Vogt,[6] and since extensively modified,[5, 25, 31] have provided simple and accurate means for studying viruses indigenous to almost every animal species.

With the new methods viruses have been isolated from the skin, respiratory tract, blood and enteric tract of a variety of animal species. Viruses isolated from the blood have been chiefly members of the arbor group and will be discussed in another paper of this series. Enteric agents have been isolated from poultry,[6, 7] cats,[10] dogs,[11] swine, [4, 18] and cattle.[20, 24, 30] General reviews of these studies have been made by Hsiung and Melnick,[15] and Kalter.[18] Special consideration will be given here to agents from chickens and cattle contrasting data from the two. Problems encountered in isolation

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and characterization of these agents will be exemplified by recent studies on bovine enteric viruses.

One of the products of animal virus studies has been the recognition that viruses related to human pathogens occur extensively in the other animal species. An early indication of this broad host range of agents was the finding of poliovirus neutralizing substances in the sera of domestic animals,[5, 15, 20] particularly cattle. While specific agents responsible for these antibodies have not been positively identified, other viruses which are neutralized by human gamma globulin have been isolated from cattle.[20] Recently Klein *et al.*[22] demonstrated that a bovine enteric virus was related to a human adenovirus. Another virus, parainfluenza 3, has been shown capable of causing disease in both man and cattle,[1] and reoviruses, first isolated from chimpanzees and from man, have now also been isolated from cattle.[29] These interrelationships raise new problems in the study of the epidemiology of human and other animal diseases and offer new opportunities for prophylactic measures.

Several papers have been published concerning enteric cytopathogenic agents in cattle. For example, Klein and Earley[21] reported the isolation of a number of these agents; while Koprowski[28] reported a single isolation of poliovirus type 1 from calf feces. Kunin and Minuse[24] isolated enteric cytopathogenic agents from cattle and found them pathogenic for suckling mice and chicken embryos although the prototypes of these agents were not neutralized by antisera for the human enteroviruses. Moll and Finlayson[27] reported the isolation of an enteric cytopathogenic agent from cattle during the outbreak of a respiratory disease but the etiological relationship of agent and disease was not established.

Certain bovine enteric viruses have been shown to be pathogenic for suckling mice[24, 28] and for chick embryo when inoculated via the amniotic sac, yolk sac, and chorioallantoic membrane.[24] Tissue cultures of calf kidney,[21, 24, 26, 27] monkey kidney,[24, 26] chicken embryonic skin, and chorioallantoic cells[24] have all been used to demonstrate cytopathic effects. HeLa cells, on the other hand, have shown no pathological changes when inoculated with certain of these agents[21, 24] and monkeys and rabbits could not be infected.[24]

Cattle enteric agents vary in their ability to agglutinate red blood cells. Five of 11 virus isolates tested by Moscovici and Maisel,[26] were capable of agglutinating bovine red blood cells at 5° to 8° C., while 3 of 11 viruses agglutinated guinea pig red blood cells. None of the 11 isolates were able to agglutinate erythrocytes, neither human 0, sheep, chicken, nor horse.

In one study on avian enteric viruses,[8] 45 of 103 rectal swabs yielded cytopathic agents in chicken kidney cell cultures. Ten of these agents pro-

duced recognizable effects on the first passage in tissue culture whereas only three agents were isolated from the same group of specimens by a single passage in embryonated eggs. After several passages in tissue culture almost half the agents acquired the capability to kill chick embryos. Thus, the primary cell cultures proved more useful for initial isolation of these agents than the whole chick embryo. Not only chick kidney cultures, but duck kidney cultures as well, were susceptible to chicken enteric viruses. However, the cytopathogenic effect was not observed in calf or monkey kidney cultures. The prevalence of these viruses in chickens showed a strong correlation with the age of the birds. In chickens 0 to 4 months old, 41 of 74 specimens examined were positive while in the 5- to 12-month-old group only 4 isolations were made from 24 samples.

Only one of the avian enteric isolates was characterized in detail.[7] Serologically, this agent was distinguishable from certain other isolates of the series and similar to others. It was neutralized by sera prepared against a laboratory strain of chick embryo lethal orphan (CELO) virus.[16] The enteric agent produced signs of central nervous system involvement when inoculated intracerebrally into baby chicks, but mice and rabbits showed no signs of infection when inoculated.

MATERIALS AND METHODS

PREPARATION OF PRIMARY CELL CULTURES

Kidney cell cultures have been used chiefly and often exclusively for the isolation of enteric viruses. There are reasons why kidney should be a first choice, but they are not sufficiently imperative that kidney should ultimately be the only system tested. Kidneys of many animals can be readily dispersed by trypsin into a suspension of single cells. These cells are relatively tolerant of cultural deficiencies but susceptible to a wide range of viruses. They are predominantly epithelial and show distinct morphologic changes when infected with many agents.

A simple method for the preparation of chicken kidney cells has been described by Hwang *et al.*[17] Fragments of kidneys from week-old chicks were incubated at room temperature for one hour in 0.25 per cent trypsin solution prewarmed to room temperature, 10 ml. being used for each pair of kidneys. At the end of the incubation period the mixture was shaken vigorously by hand for three to five minutes until pink tissue fragments dissociated. The cell suspension was then centrifuged at 800 rpm for 10 minutes, the sediment resuspended in a growth medium containing 0.5 per cent lactalbumin hydrolysate, 10 per cent calf serum, antibiotics, and a Hanks' salt solution base,[14, 15] and filtered through four layers of gauze. The filtrate was further diluted with medium to a total volume of 100 ml. for each pair of kidneys used. One ml. of the cell suspension was seeded into each tube. A dense full cell sheet usually developed in five days, at which time the culture medium was changed to a maintenance medium which contained 0.5 per cent lactalbumin hydrolysate, 0.5 per cent tryptose (Difco), and antibiotics, in Hanks' salt solution supplemented with 0.07 gm. per cent sodium bicarbonate.

Calf kidneys were prepared by a method based on that described by Bodian[5] for monkey kidney cells. Kidneys from calves under six months of age were obtained from a commercial slaughterhouse. It was not found necessary to process the kidneys immediately after the animal was slaughtered as kidneys held for as long as seven days at 4° C. were still suitable for trypsinization. The fat and capsule were stripped from the kidney, and portions of the cortex removed from the lobes with a minimal amount of medullary tissue. This tissue was cut into fragments, a few mm. in diameter, and washed in phosphate buffered saline (PBS) until the supernate was clear. It was then placed in a flask with approximately 10 volumes of 0.25 per cent trypsin in PBS

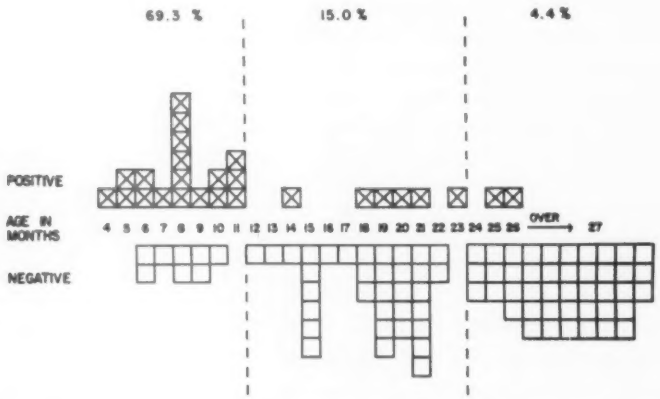

FIG. 1. Frequency of isolation of enteric cytopathogenic agents from cattle of different ages. Each square containing a cross represents an animal from which an agent was isolated. Open squares represent cattle from which no isolation was made.

and kept in a refrigerator overnight. The next morning the flask was shaken vigorously until the fragments disintegrated. The cells were then centrifuged at 600 rpm. for 10 minutes and the supernate decanted. Lastly the cells were washed in PBS and re-suspended in growth medium similar to that used for chicken kidney cells except that the calf serum concentration was increased to 15 per cent. The cells were filtered through four layers of gauze, counted and seeded at a concentration of 200,000 per ml. When a complete cell sheet formed, usually four to seven days later, the growth medium was replaced with a maintenance medium identical to that used for chicken kidney cells.

BOVINE ISOLATES

Collection of specimens. Cotton swabs were prepared on 12-inch wooden applicator sticks and used for collection of fecal samples. The swab, moist with fecal material, was immediately placed in a 15 ml. lusteroid tube containing 8 ml. trypticase soy broth and antibiotics.

FIG. 2. Bovine enteric cytopathic agents showing a sample with mixed plaque types (15 days after inoculation).

FIG. 3. Bovine enteric cytopathic agents showing three morphologically distinct plaque types. Left, type A; Middle, type B; Right, type C (9 days after inoculation).

RESULTS

Age of animal vs. isolation rate. During this study, isolations of enteric cytopathogenic agents were made in bovine kidney culture from 26 of 111 cattle tested. The highest frequency of isolations, 69.3 per cent, occurred in those less than one year old, whereas 15.0 per cent of those between one and two years old were positive, and only 4.4 per cent of those over two years old (Fig. 1).

Mixed virus populations. All but one of the 26 isolates grew readily when transferred from bovine to monkey kidney cultures and gave titers in the latter system comparable to those on calf kidney. The CPE caused by each of the isolates on both calf and monkey cultures was so similar to the others that it was impossible to distinguish differences between them on this basis. The 25 strains that grew in the monkey system all gave clear plaques under agar, but 21 of the isolates gave more than one plaque type, suggesting mixed populations (Fig. 2). Three plaque types, shown in Figure 3, predominated and were designated A, B, and C. The type A plaque developed to approximately 10 mm. in diameter in seven days when incubated at 37° C. and contained scattered surviving cells which conferred a hazy appearance to the plaque. Type B plaques developed to approximately 5 mm. in seven days and were clearly outlined. Type C plaques, on the other hand, were very small, developing to only 1 mm. in diameter in seven days, with an opaque central core usually present. Mixtures of plaque types A and B appeared in three cases, A and C in eight, B and C in six, and A, B, and C in four cases.

Since most of the young cattle carried multiple types of enteric agents at the time they were tested, neutralization tests might have ended in confusion had not the plaque purification method been available. In fact, the total number of agents may have been considerably more than the four types recognized (including one non-plaque former that grew in bovine culture). A prototype of each plaque type was purified by picking the plaque and subculturing. The purified strains were used in the serologic studies described below.

Gradocol filtration. Definitions of viruses are manifold but for practical purposes infectious agents less than 200 mμ in diameter may be referred to as viruses. To see if the bovine agents might be so designated, filtration experiments were carried out. The three cattle agents were passed through a series of gradocol membranes to determine their approximate size (Fig. 4). "A" passed all membranes tested including one with an average pore diameter of 52 mμ but showed considerable loss of titer in the finer filters.

This would indicate a diameter less than 34 mμ, a value typical of human enteroviruses. The other two agents were held back by the 52 mμ membrane but not by the 150 mμ A.F.D., suggesting diameters between 34 and 96 mμ. Since B and C were affected by the 150 mμ membrane to a degree similar to A, it is likely that their size is in the lower part of this range.

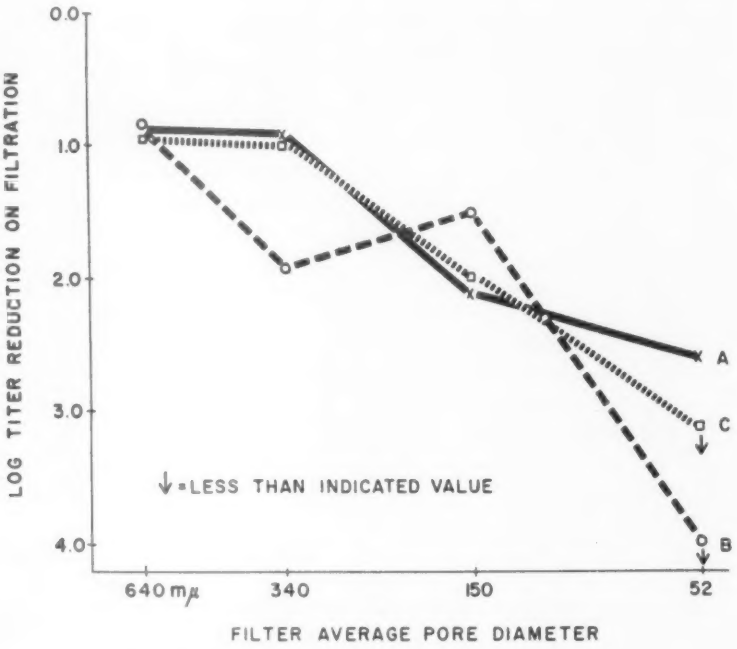

FIG. 4. Filtration data for three bovine enteric agents.

Immunization of monkeys with cattle agents. Monkeys were immunized against each of the three prototype virus strains by seven intramuscular injections approximately at one-week intervals. The plaque reduction technique was used in testing the sera and the end point taken as the highest dilution giving 80 per cent plaque reduction. All sera were heated for 30 minutes at 56° C. Serum to be used in the overlay medium was tested for inhibitory substances or, during the latter part of the study, serum was omitted entirely. No difficulty was encountered in obtaining adequate cell survival in spite of this deliberate omission.

All the animals immunized had moderate prior titers against all three viruses (Table 1), as did other normal monkeys and rabbits. It is not known if these pre-existing neutralizing substances were true antibodies, but their stability at 56° C. suggests this as a possibility. Cross neutralization tests between strains could only be carried out by comparison of pre- and post-titers. The animals immunized with A and C showed 16-fold increases in specific titer, but A stimulated antibody protection against C as well as against the homotypic agent. It is conceivable that even after plaque purification the A strain contained minute quantities of C virus,

TABLE 1. SERUM TITERS IN CROSS NEUTRALIZATION TESTS WITH ENTERIC AGENTS ISOLATED FROM CATTLE AND HYPERIMMUNE MONKEY SERA

		Titers of hyperimmune monkey sera					
		Type A		*Type B*		*Type C*	
Plaque types	*Isolate number*	*Pre-inoc.*	*Post-inoc.*	*Pre-inoc.*	*Post-inoc.*	*Pre-inoc.*	*Post-inoc.*
A	#97	16	256	16	16	16	16
B	#93	64	64	64	64	64	64
C	#48	64	2568	16	16	16	256
B	LCR4*	256	256	256	256	64	64
B, C	Moll's**	64	64	256	256	64	256

* Kunin and Minuse.[24]
** Moll and Finlayson.[27]

but when the same strain was later used to immunize goats a specific response was obtained. Immunization with B gave no effect. Thus, it was not found possible to get completely type-specific sera that would eliminate all possibility of relationships among these strains, although differences in the ratios of the pre-existing titers and the differences in the responses after immunization indicated antigenic differences existed among these viruses.

Two viruses isolated from cattle by other workers were also tested against these sera. Both were neutralized by all pre-specimens and there was no increase in titer in the post specimens except against Moll's agent in the animals immunized with C. It may be noted that the strain of Moll's agent tested gave a mixture of two plaque types, one of which was similar to that of our C strain.

Tests with human enterovirus antisera. Because of the ubiquity in various animal sera of substances capable of neutralizing the cattle agents, all human enterovirus antisera were diluted 30-fold or to a point where they

would neutralize 100 TCD_{50} of homologous virus. In this way it was possible to dilute out most of the non-specific activity. Antisera against the following enteroviruses were tested: Poliovirus 1 to 3, Coxsackie virus B, 1 to 5 as well as A9, and Echo 1 to 14. In no instance was more than 80 per cent (0.7 logs) of cattle agent neutralized by a serum dilution capable of neutralizing 99 per cent (2.0 logs) or more of homotypic virus. Thus, while none of the cattle agents was neutralized by sera to human enteroviruses to an extent comparable to the homologous titer, these techniques did not eliminate entirely the possibility that the cattle agents may have represented S (specific) phase variants[19] of human virus prototypes.

TABLE 2. CATTLE SERA* SHOWING NEUTRALIZING ACTIVITY AGAINST 100 TCD_{50} OF POLIOVIRUSES, TYPES 1, 2, 3

Age of cattle in months	*Number of animals tested*	*Number of sera with neutralizing activity against polioviruses*		
		Type 1	*Type 2*	*Type 3*
4-11	26	1	1	0
12-23	40	4	7	0
24-	45	8	20	1
Totals	111	13	28	1

* Cattle sera heat inactivated and diluted 1 : 10.

Tests with cattle sera against human viruses. Neutralizing activity was found in cattle sera against all three poliovirus types. Type 2 was neutralized most frequently and Type 3 least often (Table 2). There was an increased prevalence of neutralizing activity with increasing age of the animals, consonant with its derivation from an antigenic stimulus.

Studies were conducted on paired sera of eight animals that had been tested for enteric cytopathogenic agents (Table 3). The initial sera were tested at dilutions of 1 :2 to 1 :16, and the subsequent sera were tested at dilutions of 1 :8 to 1 :64. Two of four animals developed Type 1 antibodies and one of four developed Type 2 antibodies during the period of study.

Four other cattle sera, drawn two months after the fecal samples were taken, were selected for neutralization studies with Coxsackie and ECHO viruses. All three plaque types had been isolated from each of these animals. One serum had neutralizing activity against ECHO 2 and Coxsackie B5, another against ECHO 2 and Coxsackie B2, and a third against Coxsackie B2 only. The fourth specimen showed no neutralizing activity

for any ECHO or Coxsackie virus type tested. If the cattle agents were related to the human virus, antibodies against them should be effective against the broad phase human virus prototypes regardless of the phase of the infecting agent. Since one animal which had been infected with all three bovine plaque types possessed no antibodies to the human agents, some members of each plaque type must have been unrelated to any human agent tested. However, it has not been established that all isolates of one plaque type are related, and hence the positive reactions found in the other animals may have been elicited by certain of the viruses isolated.

TABLE 3. CHANGES IN POLIOVIRUS NEUTRALIZING TITER IN SERUM FROM CALVES

Animal number	*Plaque types isolated*	*Age of animal (months)*	*Interval between bleedings (months)*	*Antibody titers to polioviruses*	
				Initial	*Subsequent*
				Type I	
84	AB	4	2	<2	<8
90	AC	6	2	<2	16
93	ABC	8	2	8	<8
97	AB	8	2	4	32
				Type II	
21	None	18	6	16	16
48	AC	14	6	<2	8
97	AB	8	2	16	8
108	ABC	11	2	2	<8

SUMMARY

The use of primary cell cultures for the isolation of enteric viruses from domestic animals has opened a new field of investigation. The results, thus far, indicate that the intestinal tracts of cattle and poultry are fertile fields for virus growth. The number of isolations and their apparent heterogeneity make the task of identification and classification a major undertaking, but, aside from their implications in veterinary medicine, these agents cannot be ignored in complete studies of the epidemiology of human infections.

Of 26 isolations of enteric cytopathogenic agents made from 111 cattle fecal samples, 69.3 per cent of the specimens from animals 4 to 11 months of age were positive, 23.1 per cent from animals 12 to 23 months old, and 7.6 per cent from animals over 24 months of age.

Cytopathologic effect (CPE) in both calf and rhesus monkey *(Macaca mulatta)* kidney cells was produced by 25 of the 26 isolates. One isolate produced CPE in calf kidneys only. The cattle agents that produced CPE in monkey kidney cells also produced plaques on these cells. The plaque method revealed that 84 per cent of the isolates contained a mixture of agents. Three plaque types predominated.

Serological tests using plaque-purified strains revealed differences between the three prototypes. Neutralizing activity for these agents was found widely distributed in monkey and rabbit sera, but titers were significantly lower than homologous values in 23 serum pools from monkeys hyperimmunized with different human enteroviruses. Certain sera from cattle carrying these agents possessed neutralizing activity for the poliovirus group, Coxsackie virus types B2 and B5, and for ECHO virus type 2. However, there was no apparent correlation between the type of cattle agent isolated and the type of human agent neutralized.

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APPLICATIONS OF PRIMARY CELL CULTURES IN THE STUDY OF ANIMAL VIRUSES**

II. Variations in Host Responses to Infection by Certain Arthropod-borne Viruses†

In studies of the susceptibility of various primary tissue culture systems to arthropod-borne virus infection, it was found that host cell reactions induced by certain members of this group of viruses may take a variety of forms under the influence of various conditions.[5, 9, 10, 20] Thus, a single virus strain may stimulate a different type of cellular response in each of two equally susceptible culture systems. For example, when Semliki forest virus was grown in chick embryo and rhesus monkey kidney monolayers, each culture system responded differently even though virus multiplication was proceeding at about the same rate in both as shown by the titration of infected tissue culture fluids in mice (Fig. 1). When these infected cell monolayers were examined microscopically, it was noted that the chick embryo cells were apparently undergoing rapid, irreversible, degenerative changes two days after virus inoculation, while only limited foci of somewhat questionable cytopathic changes were observed in the rhesus cultures inoculated under similar conditions. Plaques would form under an agar overlay, however, at the same rate in both types of cultures and independently of the cytopathic responses observed in the parallel fluid cultures.[27] It is, therefore, the purpose here to review such types of variations in cell culture responses induced by selected members of the arthropod-borne virus sero-groups.[4]

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VIRAL CYTOPATHOGENICITY IN FLUID CULTURES

Cytopathic effects (CPE) detected microscopically have been most commonly used as the criterion for arthropod-borne virus infection in tissue culture.[1, 5, 6, 19, 20, 23, 24] An analysis of such responses of certain primary cell cultures to a number of arthropod-borne viruses was undertaken to determine the suitability of this reaction for determining host cell susceptibility. CPE produced in cultures as the result of arthropod-borne virus infection have generally taken the form of a progressive granulation, rounding, clumping, and ultimate dislodgement of cells from the surface of the culture vessel. Examples of some of these cellular changes are illustrated in Figure 2. Whatever the origin of the cells in culture or the type of agent, the cytopathic responses are quite similar and comparatively distinct as compared with groups of other cytopathic agents such as the enteroviruses,[18] adenoviruses,[22] or measles virus.[7]

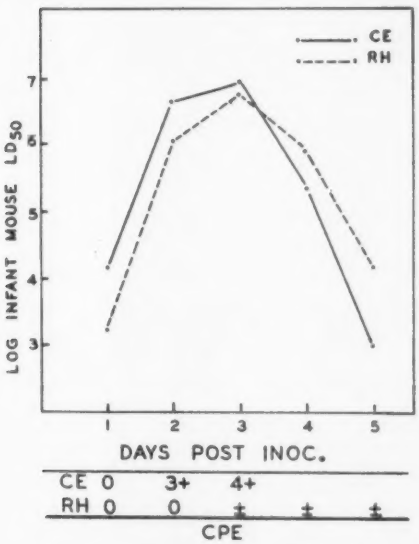

FIG. 1. Growth curves of Semliki forest virus in chick embryo (CE) and rhesus monkey kidney (RH) tissue cultures with accompanying cytopathic effects (CPE).

In Table 1 are summarized in a qualitative fashion, the cytopathic reactions induced by 19 arthropod-borne viruses in primary cell cultures prepared from various mammalian and avian tissues. It should be emphasized that these reactions are based on the inoculation of cell cultures with varying dilutions of virus prepared from infected mouse brain. No attempts were made to adapt virus strains to specific culture systems. In instances where virus multiplication proceeded without accompanying cellular degeneration detectable by microscopic examination, the reaction was recorded as negative.

Primate tissue cultures

As can be noted in Table 1, cytopathic reactions in primate kidney cell culture (human kidney cultures included) were induced by only about one-

TABLE 1. ARTHROPOD-BORNE VIRUS CYTOPATHIC EFFECTS (CPE) MEASURED IN FLUID TISSUE CULTURES PREPARED FROM VARIOUS ANIMAL KIDNEY AND EMBRYONIC TISSUES

		Primate					*Non-primate mammal*							*Avian*		
Virus sero-group	*Mouse brain virus*	Human kid.	Rhesus monkey	Patas monkey	Cebus cap. monkey	Baboon	Dog	Pig	Lamb	Rabbit	Guinea pig	Hamster	Ferret	Chick embr.	Pekin duck	White Leg. chicken
A	E. eq. enceph	+	+	+	+	0	+	0	0	+	+	+	+	+	+	+
	W. eq. enceph.	0	I	0	0	0	+	0	0	0	+	+	0	+	+	+
	Sindbis	0	0	0	0	0	0	0	0	0	+	+	0	+	I	+
	Semliki forest	0	0	0	+	0	+	+	0	0	+	+	0	+	+	+
	Mayaro	0	0	+	0	0	0	0	0	0	+	+	0	I	+	0
	Middleburg	0	0	0	0	0	0	0	0	0	0	+	0	0	0	0
B	Japanese B	0	0	0	0	0	+	0	0	0	0	+	0	0	0	+
	St. Louis	0	0	0	0	0	0	0	0	0	0	+	0	0	0	+
	Ntaya	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0
	Ilheus	0	+	+	0	+	+	+	0	0	+	+	0	0	0	0
	West Nile	0	0	0	0	0	+	0	0	0	0	+	0	0	+	0
	Bussuquara	0	0	0	0	0	0	0	0	0	0	+	0	0	I	0
C	Oriboca	I	0	0	0	0	+	0	0	0	+	0	0	0	0	0
	Caraparu	0	0	0	0	0	0	0	0	0	0	0	+	0	0	0
	Murutucu	I	0	0	0	0	0	0	0	0	0	0	+	0	0	+
	Marituba	0	0	0	0	0	0	0	0	0	0	0	0	0	0	□
	Apeu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
Ungr.	Bwamba		0	+	0	0	+	0	0	0	+	+	0	0	+	0
	Bunyamwera		+	+	+	0	+	0	+	+	+	+	+	+	0	0

+—Virus induces CPE.
0—Virus does not produce CPE in first or subsequent tissue culture passages.
I—Inconsistent CPE.

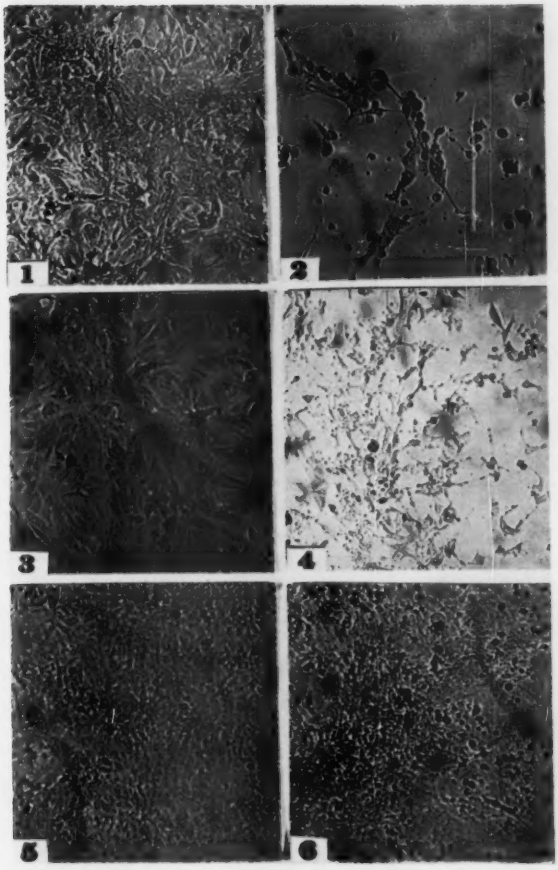

FIG. 2. Cytopathic effects induced by certain arthropod-borne viruses in primary cell cultures. (i) Uninoculated rhesus monkey kidney culture; (ii) Eastern equine encephalomyelitis (EEE) infected rhesus culture, three days after inoculation; (iii) Uninoculated guinea pig kidney culture; (iv) Mayaro infected guinea pig kidney culture, two days after inoculation; (v) Uninoculated duck embryo culture; (vi) West Nile infected duck embryo culture, five days after inoculation.

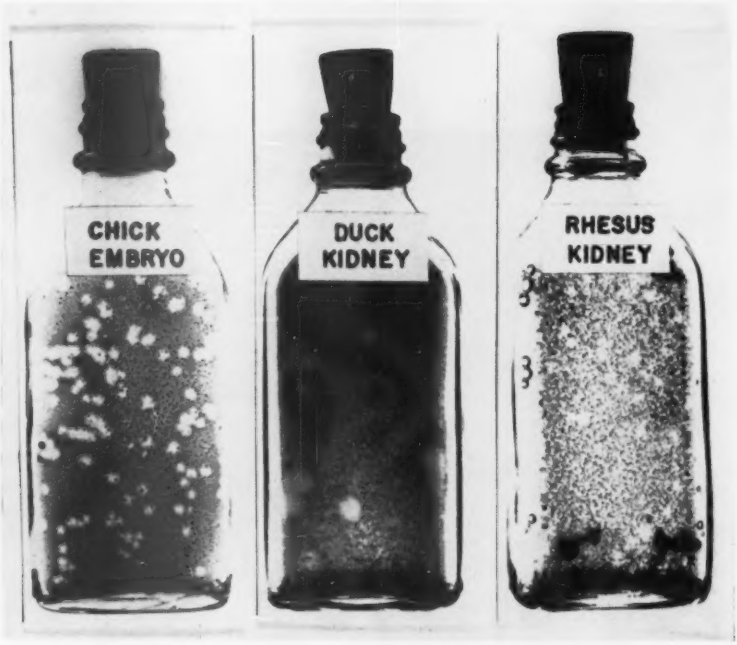

FIG. 3. Plaques of Middelburg virus (group A) in chick embryo 2 days after inoculation, in Pekin duck kidney 4 days after inoculation, and in rhesus monkey 4 days after inoculation.

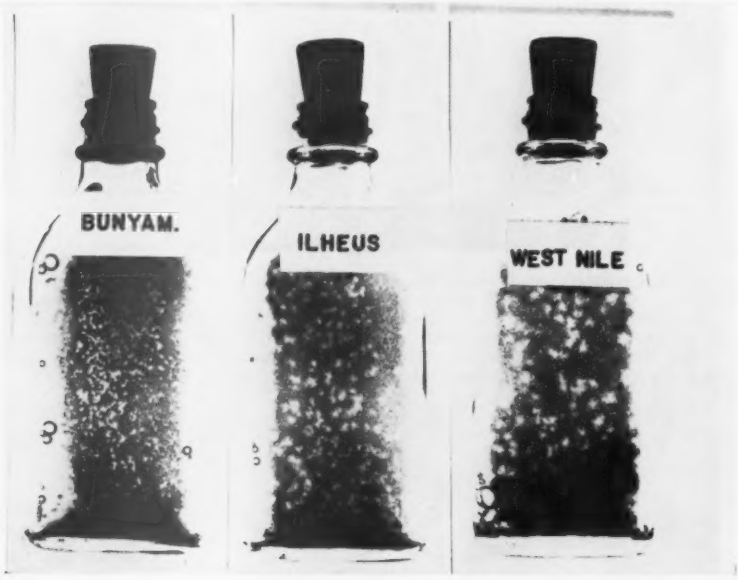

FIG. 4. Dog kidney cultures inoculated with Bunyamwera virus (ungrouped), 9 days after infection. Ilheus virus (group B), 9 days after infection, and West Nile virus (group B), 11 days after infection.

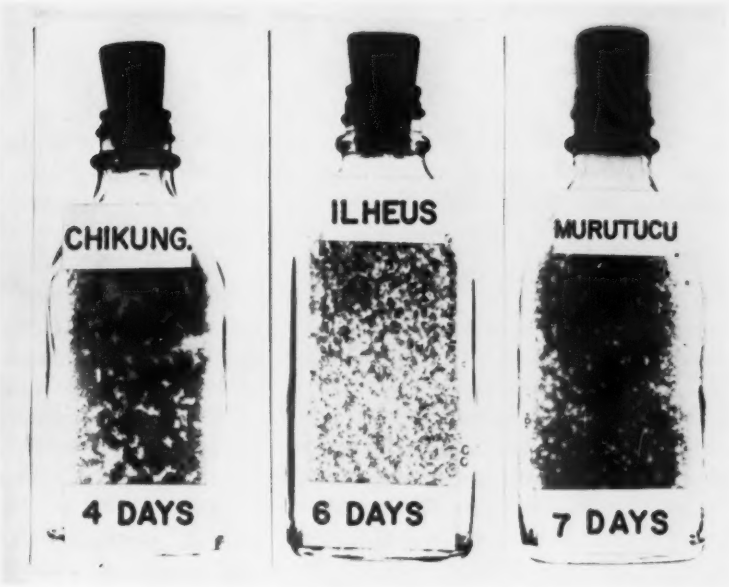

FIG. 5. Chickungunya (group A), Ilheus (group B), and Murutucu (group C) virus plaques in rhesus monkey kidney cell cultures.

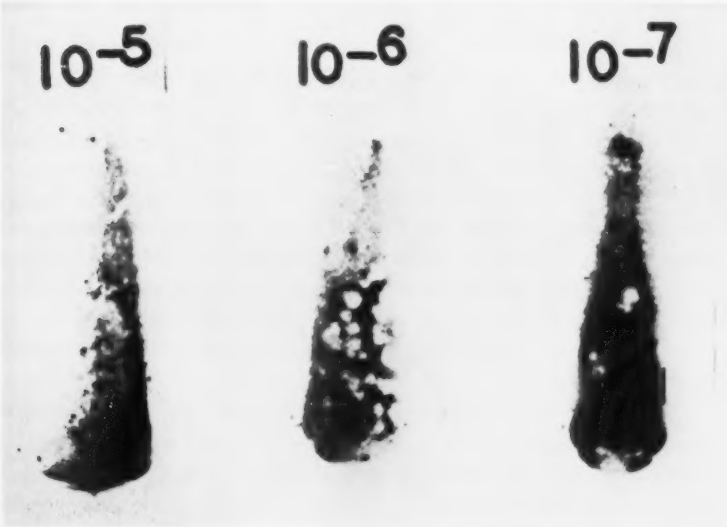

FIG. 6. Plaques of Semliki forest virus at various dilutions (10^{-5}, 10^{-6}, 10^{-7}) in chick embryo tube cultures, two days after inoculation.

half the viruses studied (9 out of 19). It is conceivable, however, that many viruses recorded as non-cytopathic in the first or subsequent passages could have been adapted to certain primate tissue cultures and ultimately be classified as cytopathic agents.[9, 11]

Non-primate mammalian tissue cultures

Kidney cultures prepared from seven animal species were collectively capable of supporting multiplication of 16 out of 19 viruses with accompanying cytopathic reactions (Table 1). Hamster and guinea pig kidney cultures were generally more responsive to infection than tissues obtained from other animals of this group, as was similarly noted by other investigators.[5, 16] Kidney tissue from porcine fetuses was found previously to be suitable for the propagation of Japanese B encephalitis virus using the cytopathic index.[17] Cultures prepared in this laboratory from adult kidney tissues of this same animal species, however, did not show evidence of CPE following Japanese B virus inoculation. In addition to those non-primate mammalian species listed, primary cell cultures prepared from whole mouse embryos and cat and calf kidney tissues were also inoculated, but recognizable CPE were not evident with any of the 19 viruses tested.

Avian tissue cultures

From an earlier report,[28] it would appear that avian embryonic tissues, particularly the developing chick embryo, would generally be the most promising for propagation of the arthropod-borne viruses. More recently, reports concerning a broader application of this tissue type for supporting growth of numerous arthropod-borne viruses have appeared.[18, 21, 24, 25, 29, 30] The cytopathic spectrum as shown in Table 1 for cultures derived from chick embryos and from kidney tissues of Pekin ducks and white Leghorn chickens were generally more responsive when infected with the sero-group A rather than with the group B viruses, though certain group B viruses did induce CPE in duck and chicken kidney cultures.

PLAQUE-TYPE REACTIONS UNDER AGAR OVERLAY

Variations were evident, in many instances, in the rate of plaque development and the size, shape, and texture of plaques produced by various viruses when grown in cell cultures prepared from tissues of chick embryos, Pekin ducks, rhesus monkeys, or dogs. Middelburg virus, for example, produced clear, circular plaques in cultures from avian tissues but hazy, irregular

plaques in rhesus monkey kidney cultures (Fig. 3). Bunyamwera virus, on the other hand, formed minute almost pin-point type plaques in dog kidney cultures while Ilheus and West Nile viruses, grown in the same culture type and for the same period, produced larger plaques with clear

TABLE 2. COMPARISON OF CYTOPATHIC AND PLAQUE REACTIONS FOR SELECTED ARTHROPOD-BORNE VIRUSES OF THE MAJOR SERO-GROUPS

	Cellular changes in primary tissue cultures					
	Chick embryo		*Duck kidney*		*Rhesus monkey*	
Viruses	*CPE*	*Pl.*	*CPE*	*Pl.*	*CPE*	*Pl.*
Group A*						
EEE (WEE, VEE)	+	+	+	+	+	+
Sindbis (Semilki)	+	+	+	+	0	+
Mayaro	I(5da)	+	+	+	0	+
Middelburg	0	+	0	+	0	+
Chickungunya	0	0	+	+	0	+
Group B**						
JBE (Buss., SLE)	0	0	0	+	0	0
WN (Zika, Uganda S)	0	0	+	+	0	0
Ntaya			+	I	0	0
Ilheus	0	0	0	0	+	+
Group C‡						
Marituba (Oriboca, Apeu, Murutucu, Caraparu)	0	0	0	0	I	+

* Plaques first appear in 2-3 days (except Mayaro).
** Plaques first appear in 4-6 days.
‡ Plaques first appear in 5-7 days.
+—Plaques or CPE evident.
0—No CPE or plaques evident.
I—Inconsistent CPE or plaques.

centers but with irregular or serrated edges (Fig. 4.) Generally, however, most of the arthropod-borne viruses, regardless of the sero-group to which they belonged, induced similar types of plaques in rhesus cultures (Fig. 5). The plaque variations which did occur were usually not found to be sufficiently distinctive for each virus or sufficiently characteristic for each sero-group for purposes of virus differentiation, as has been done with the enteroviruses.[38]

CYTOPATHIC EFFECTS VS. FORMATION OF PLAQUES

A comparative study of virus-induced cytopathic effects in fluid cultures and plaque reactions under an agar overlay was undertaken. In these studies, a total of 21 viruses was examined in chick embryo, Pekin duck kidney, and rhesus monkey kidney primary cell cultures. The results (Table 2) show that the incidence of plaque formation far exceeded the inducement of cytopathic reactions by these viruses. It could be demonstrated,

TABLE 3. COMPARATIVE TITRATIONS OF REPRESENTATIVE ARTHROPOD-BORNE VIRUSES UTILIZING CYTOPATHIC, PLAQUE, AND MOUSE INFECTIVITY ENDPOINTS

		Tissue culture titration/0.1 ml.		
Virus strain	*Tissue culture system*	*Log TCD_{50}*	*Log Av. PFU*	*Infant mouse Log LD_{50}/1.0 ml.*
EEE	CE	7.1	8.8	7.4
Sindbis	CE	6.4	8.0	7.2
Semliki	RMK	6.5	7.6	6.9
Ntaya	PDK	4.5	5.7	5.4
Ilheus	RMK	5.4	6.5	5.7
West Nile	PDK	6.6	7.9	6.8

CE—Chick embryo.
RMK—Rhesus monkey kidney.
PDK—Pekin Duck kidney.

in most instances, that if a virus was capable of multiplying in any single culture system, plaques but not necessarily CPE would result. Furthermore, plaque reactions have been found to be consistent from experiment to experiment as opposed to the erratic and at times questionable CPE in fluid cultures.

In addition, the plaque method has been found to be usually more sensitive for virus assay than cytopathic reactions or mouse infectivities. Examples of parallel titrations utilizing such cytopathic, plaque, and mouse lethality endpoints are shown in Table 3. Virus titers obtained in infant mice were usually about equal to those obtained in fluid cultures. The plaque method, however, whether performed in bottles[10] or tubes[8] (Fig. 6), was found to be 10 and often 100 times more sensitive than either of the other methods (certain group C viruses excepted).[10]

Of the group A viruses, Eastern, Western, and Venezuelan equine encephalomyelitis (EEE, WEE and VEE), Sindbis, and Semliki forest

viruses all produced CPE in fluid chick embryo cultures as well as plaques under agar two-three days after infection (Table 2). Mayaro virus-induced CPE were evident in chick embryo fluid cultures only when high multiplicities of virus were inoculated, and plaques appeared only when cultures were kept five to six days after infection. Fluid chick embryo cultures also supported multiplication of Middelburg virus, even though CPE were usually absent, but plaques were regularly demonstrable in agar overlaid cultures. All of the group A viruses studied formed plaques in Pekin duck embryo and rhesus monkey kidney cultures, but again in many instances, CPE in parallel fluid cultures were questionable or not detectable.

Pekin duck kidney or duck embryo cultures were found to be quite suitable for supporting multiplication of most of the group B viruses with plaques appearing in five-six days after inoculation and induced by Japanese B, St. Louis, Ntaya, West Nile, and Bussuquara (Table 2). Japanese B, St. Louis, and Bussuquara failed to induce recognizable cytopathic changes in fluid cultures, though within the same virus serogroup, distinct CPE were produced by West Nile, Zika and Uganda S viruses. Ntaya virus was capable of multiplying in duck cultures with resulting cytopathic changes, but plaques under agar overlay were difficult to demonstrate. Ilheus, on the other hand, produced both CPE and plaques in rhesus but not in duck cultures.

Of the group C viruses studied, all produced plaques in five-seven days in rhesus overlaid cultures with variable or no cytopathic alterations in parallel fluid cultures (Table 2). Rhesus monolayers, however, were found to have a low degree of susceptibility to Apeu and Caraparu although plaques could be demonstrated regularly when high multiplicities of virus were inoculated.[10]

In general group A viruses were found to multiply in both avian (chick embryo and Pekin duck kidney or embryo) and the primate (rhesus monkey kidney) cell systems with few exceptions. Group B viruses could be propagated in Pekin duck kidney or Pekin duck embryo cultures, but not in chick embryo or rhesus monkey kidney (except Ilheus), and the group C viruses produced only plaques in rhesus monkey kidney cell cultures. Thus, the grouping patterns based on serology[4] and the grouping patterns evident by virus host cell range may be quite similar with respect to certain arthropod-borne viruses. Furthermore, the grouping or sub-grouping of viruses by their tissue culture susceptibility spectrum as previously proposed[12, 14, 15] may be an important technique for further categorizing the ever-expanding members of arthropod-borne virus family.

SUMMARY

During attempts to investigate the responses of 15 different primary cell cultures to infection by certain arthropod-borne viruses, two (Pekin duck and rhesus monkey) or more culture systems were capable of supporting multiplication of essentially all the virus strains tested. Arthropod-borne virus plaque formation in agar overlaid cultures was found to be a better index of tissue culture susceptibility to infection than virus induced CPE in fluid culture systems. In addition, virus assay by the plaque method was usually found to be more sensitive than assays performed by either the cytopathic or infant mouse lethality endpoints. The morphology of plaques produced by specific viruses varied depending upon the cell culture system used, and it was not possible to differentiate the arthropod-borne viruses by the types of plaques formed. However, sero-group differentiation could be made to some extent with certain viruses on the basis of host cell susceptibility range.

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APPLICATIONS OF PRIMARY CELL CULTURES IN THE STUDY OF ANIMAL VIRUSES**

III. Biological and Genetic Studies of Enteric Viruses of Man (Enteroviruses)†

Since the discovery by Enders, Weller, and Robbins in 1949[9] that poliovirus could multiply in cultures of non-nervous tissues, cell culture techniques have been used extensively in the studies of enteroviruses[3] for the last decade. The accumulated literature reveals that the usefulness of these culture methods is many-fold and may include virus isolation, virus multiplication studies, and serological diagnoses of disease, as well as epidemiological investigations. A review of tissue culture techniques in the study of enteroviruses has been published in detail by Weller[44] and by Melnick.[34] This discussion will deal with a few biological characteristics of certain enteroviruses in primary cell cultures and the applications of these culture techniques in genetic studies of animal viruses.

BIOLOGICAL CHARACTERISTICS OF ENTEROVIRUSES

Host cell specificity

In searching for a uniformly susceptible host system which can be easily obtained for the study of the entire family of enteroviruses, primary kidney cell cultures derived from a variety of primates and non-primates were tested. In the primate group, human kidney tissues, as well as kidney cells from Old World monkeys, baboons of Africa and Asia, and New World monkeys from South America were grown in culture. In the non-primate series, kidneys of domestic and laboratory animals were used. The results for the subsequent discussion on host cell specificity are shown in Figure 1.

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Primate kidney cell cultures. It is apparent that human kidney and rhesus *(Macaca mulatta)* cell cultures were sensitive to infection by all the enteric viruses tested. Patas *(Erythrocebus patas)* cells were highly susceptible to the three types of poliovirus, group B Coxsackie and group B of ECHO (types 7, 10, and 12) viruses but resistant or partially resistant

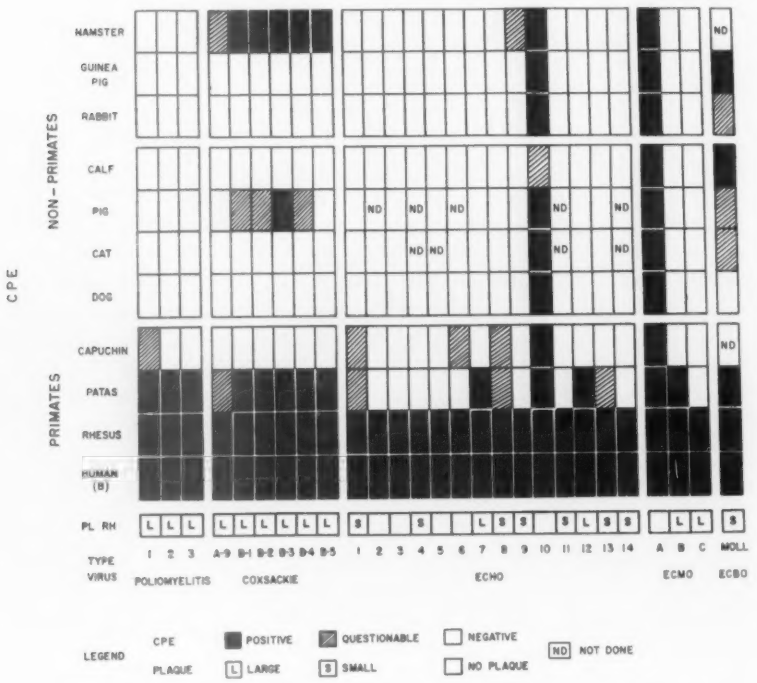

FIG. 1. Comparative susceptibility of kidney cell cultures to enteric viruses.

to Coxsackie A 9 and group A of ECHO viruses.[19, 10] Capuchin *(Cebus capucina,* a New World monkey) kidney cultures, on the other hand, were found susceptible only to ECHO-10 among the enteroviruses tested, as has been observed by Ramos-Alvarez and Sabin.[27, 28] In addition, ECHO-10 was the only one of the enterovirus group which caused cytopathic changes in patas cells but was unable to produce plaques on rhesus or on patas monolayers.[28]

Non-primate kidney cultures. Of the non-primate kidney cultures utilized, pig kidney cells originally showed cytopathic effects (CPE) when infected with Coxsackie B-1, 2, and 4, but upon repeated passage of these viruses, these effects were gradually lost. In contrast, Coxsackie B-3 produced consistent CPE upon repeated subculture. Barron and Karzon[1] reported that Coxsackie A 9 and Coxsackie B 1-5 produced CPE in hamster kidney cultures, but we have found that hamster cells were susceptible only to the group B Coxsackie viruses.

Among the ECHO viruses tested in this laboratory, ECHO-10 caused typical CPE in kidney cultures derived from dog, cat, pig, rabbit, guinea pig, hamster, and to some extent calf.[28] Similar changes with this agent in pig kidney cultures have been noted by Guerin and Guerin.[12] ECHO-10 virus titers obtained from non-primate cultures were essentially the same as those of the primate cultures as found in our previous studies[28] and as reported by Barron and Karzon in their studies using hamster kidney cultures.[1]

Two strains of Group A enteric cytopathic monkey orphan (ECMO) viruses[27, 28] were found to produce CPE in all primate and non-primate tissue cultures tested, while group B and group C of ECMO viruses grew only in primate cultures. The enteric cytopathic bovine orphan virus (ECBO) isolated by Moll and Finlayson[25] caused cytopathic changes only in some of the non-primate cultures, and in all primate cultures tested.

From these data, it is evident that ECHO-10 is distinct in its host cell range and has been demonstrated to differ from those of the other members of enterovirus group. Its ability in common with certain simian viruses to multiply in non-primate kidney cultures adds support to the notion that it should be removed from the ECHO virus group, and recently the name "Reoviruses" was proposed for ECHO-10 and the related viruses.[29]

Human kidney cell cultures. Human kidney (HK) cultures supported multiplication of poliovirus types 1, 2, and 3 (both virulent and attenuated strains) with accompanying cellular degenerative changes in kidney cultures derived from adult and infant tissues obtained at autopsy. There were no significant differences in attenuated and virulent poliovirus plaque titers or virus yields, although strains of attenuated poliovirus produced smaller plaques than virulent strains on HK cultures. Human kidney cell culures derived from premature babies were highly sensitive to infection by Coxsackie and ECHO viruses and displayed distinct cytopathic changes similar to those produced by the polioviruses. Cultures prepared from certain adult kidney tissues, however, did not show these cytopathic responses when inoculated with Coxsackie A 9, Coxsackie B 1-5 or ECHO viruses (except ECHO virus types 7 and 10). Many of the Coxsackie and ECHO viruses multiplied and persisted in the cultures derived from adult tissues

TABLE 1. ISOLATION OF ECHO VIRUSES IN RHESUS MONKEY AND HUMAN KIDNEY CULTURES

Material	Days after inoc.	CPE at different virus dilutions									Virus identified
		Rh			HK(A)			HK(B)			
		Undil.	10^{-1}	10^{-2}	Undil.	10^{-1}	10^{-2}	Undil.	10^{-1}	10^{-2}	
Stool #C	4	0	0	0	0	0	0	0	0	0	ECHO-2
suspension	10	+++	+++	+	0	0	0	+++	++	+	
Stool #WB	4	0	0	0	0	0	0	+	0	0	ECHO-11
suspension	10	±	0	0	0	0	0	+++	+++	++	

Legend: Rh Rhesus monkey kidney cultures.
HK (A) Human cultures derived from adult kidney tissues.
HK (B) Human cultures derived from infant kidney tissues.

± Questionable.
+ A few cells degenerated.
++ ½ of the cell sheet degenerated.
+++ ¾ of the cell sheet degenerated.
++++ Complete degeneration.

although cellular degeneration was incomplete and not readily recognizable.[20]

The sensitivity of rhesus and human kidney cultures for the primary isolation of ECHO 2 and ECHO 11 from human stools is shown in Table 1. Rhesus monkey kidney cultures and cells derived from premature baby kidneys were equally satisfactory for the isolation of ECHO virus type 2 from stool suspensions; adult human kidney tissues were not satisfactory for this purpose. HK cultures from infants were highly sensitive for isolating ECHO 11 (WB) virus from stool suspensions, whereas rhesus cells were less susceptible to infection by this particular strain of ECHO virus. Figure 2 illustrates the CPE of ECHO-11 (WB) virus in rhesus and infant kidney cell cultures. Extensive cellular destruction was observed in the HK culture (derived from premature baby kidney tissue) eight hours after infection, whereas only small foci of degeneration occurred in rhesus cultures 48 hours after inoculation. It is concluded, therefore, that in a search for a universally sensitive tissue culture system for the isolation of unrecognized etiological agents of human viral infections, human cell cultures appear to be the most desirable.

Plaque characterization

The development of the plaque method by Dulbecco[7] enhanced the versatility of tissue culture techniques for studying animal viruses. However, in order to demonstrate plaques of slow-growing viruses, it was necessary to adapt Dulbecco's technique to stoppered prescription bottle cultures in which kidney cells were stable under agar for two to three weeks or longer.[17] Thus, the need for the humidified incubator and CO_2-air mixture was eliminated.

As reported in the two preceding papers, some distinctive differences in plaque patterns were noted when propagating bovine enteric viruses or arthropod-borne viruses in primary cell cultures.[14, 15, 20] Past studies[18] on the enteroviruses have shown that poliovirus types 1, 2, and 3 produced large, circular plaques with clear centers and sharp boundaries on rhesus monolayers within four or five days after inoculation, with the plaques increasing in size thereafter as shown in Figure 3. Coxsackie virus plaques were also round, resembling those of the polioviruses except for their delayed appearance, usually about six to seven days after virus inoculation, and healthy cells stained with neutral red could be seen within the area of Coxsackie virus-induced cellular degeneration. This was especially marked at the periphery of the plaques of Coxsackie A 9 (Fig. 3). ECHO virus plaques, which also are shown in Figure 3, were smaller and more irregu-

lar in shape than poliovirus plaques, and their boundaries were diffuse. Exceptions in this pattern were ECHO types 7 and 12 which formed plaques indistinguishable from those of the polioviruses (Fig. 3).

Mixed infections were easily and quickly recognized by the use of the plaque technique. However, if this method is used for virus isolation, fluid cultures should also be inoculated in order to detect ECHO viruses which do not produce plaques (ECHO virus types 2, 5, and 6) but induce CPE in fluid cultures. We have also observed recently that certain ECHO viruses produced plaques under agar overlay but were incapable of producing cytopathic changes in parallel fluid cultures.[35] A similar phenomenon was originally described in tissue culture studies of the arthropod-borne virus group.[18]

FACTORS INFLUENCING VIRUS MULTIPLICATION UNDER AGAR OVERLAY

Components in agar overlay medium

Concentration of sodium bicarbonate. In confirmation of reports by Vogt *et al.*[41] we have found that in bottle cultures the concentration of bicarbonate in the agar overlay affects plaque formation of attenuated strains of poliovirus.[21] The two concentrations of bicarbonate, namely 0.075 and 0.45 gm per cent, were used in this laboratory as markers for the differentiation of virulent and attenuated poliovirus strains.

Amino acids, serum, and other proteins. Dubes[4] found that development of polio plaque was evident in cultures when the overlay medium was enriched with the addition of L-cystine. Rappaport[33] showed that in the presence of certain concentrations of glycerine, using a protein-free synthetic overlay medium, poliovirus plaque numbers were reduced, and the rate of plaque development was retarded. Arginine was found to be required for adenovirus plaque production.[3]

Takemoto and Habel[40] found that 2 per cent horse serum from certain horses when added to the overlay caused inhibition of 90 per cent of the plaques produced by poliovirus type 1. This serum "inhibitor" was heat stable, non-dialyzable, and did not neutralize the virus. Plaque formation by poliovirus types 2 and 3 and Coxsackie A 9 virus was apparently unaffected by the "inhibitor." In contrast, plaque formation of ECHO virus types 8 and 18 was accelerated by the addition of lactalbumin hydrolysate in the overlay medium.[36]

Neutral red and light effect. Photosensitization of tissue cultures in the presence of neutral red was noted by Green and Opton[11] and by Klein and

Goodgall.[29] The former authors found that monkey kidney cultures inoculated with poliovirus type 1 produced smaller and fewer plaques when the overlaid medium contained neutral red and were exposed to light. This photodynamic action of neutral red in cell cultures was also noted by Gouchenour and Baron.[30]

Temperature of incubation

Dubes[8] and Dubes and Wenner[9] have noted that when poliovirus multiplied at 23° C., a markedly diminished virulence in monkeys was evident. Recently, Lwoff and Lwoff[31, 32] have found that virulent strains of poliovirus grew readily at 39°-40° C., while multiplication of attenuated strains was greatly reduced at these temperatures. Yoshioka *et al.*[48] in our laboratory have confirmed this phenomenon, and, in addition, Yoshioka has shown that plaques produced by attenuated poliovirus were inhibited at high temperatures, namely 39°-40° C., but the virulent strains produced plaques equally well at 35° C., 37° C., and 39° C., although the plaques produced at 39° C. were smaller.

Cell cultures

It was noted by Frothingham[9] that susceptibility of fluid cultures of human amnion cells to a strain of poliovirus type 2 was increased as the cultures aged. In our hands we have found that younger cultures with compact cell sheets were more sensitive to virus infection under an agar overlay even though variation in plaque size and numbers was evident when different lots of cultures were used. In addition, kidney cells derived from young individuals, especially from embryonic tissues, seemed to be far more sensitive to viral infection than kidney cells derived from adults.[38] Furthermore, smaller plaques were obtained in HK cultures inoculated with poliovirus of attenuated strains than the virulent ones as shown in Figure 4, although the yields of the virus were not very different.

GENETIC STUDIES OF COXSACKIE A 9 VIRUS[37]

Plaque morphology and host cell range have been used as markers for genetic studies with bacterial viruses,[16] and apparently there are many advantages in using such markers for genetic studies in animal viruses.

Adaptation and plaque size variation of Coxsackie A 9 virus

It was shown in the host cell specificity section of this paper that patas monkey kidney cells were found quite resistant to Coxsackie A 9 virus

infection. Three attempts to propagate Coxsackie A 9 virus serially in non-irradiated patas cells were unsuccessful (Table 2). In contrast, when serial passages were made in x-irradiated patas cultures, virus yields increased at each passage and cytopathic changes became most prominent by the 5th passage.[24] In Table 3 virus populations in irradiated patas fluid cultures during the course of serial passages are analysed and variations in plaque size noted during the process of these serial transfers are ilustrated. Small plaque-type virus (9.3 per cent) emerged after the second

TABLE 2. SERIAL TRANSFERS OF COXSACKIE A-9 VIRUS IN PATAS CULTURES

	Virus titer log PFU (Rh) per ml.					
	Experiment I		*Experiment II*		*Experiment III*	
No. passages	*Non-irrad.*	*Irrad.*	*Non-irrad.*	*Irrad.*	*Non-irrad.*	*Irrad.*
0*	8.4	9.1	8.5	8.5	8.5	8.5
1	7.4	8.1	7.4	7.0	7.4	7.4
2	5.6	7.1	5.9	6.4	5.9	6.6
3	4.7	7.5	4.4	5.5	4.4	5.7
4	1.6	7.4	2.2	6.5	2.2	5.2
5	0	7.8	1.7	7.2	2.0	4.8
6	0	7.8	0	7.2	0	4.6
7			0	7.3	0	5.2
8			0	7.6	0	5.3

* Original inoculum.

passage in the irradiated patas cells, and 100 per cent small plaques were produced by the sixth tissue culture passage. The identity of the adapted virus was confirmed by neutralization with antiserum from rabbits immunized with a prototype Coxsackie A 9 virus strain.

Plaque type reversion in mice

Reversion of the adapted A 9 virus was noted after one mouse passage. Brain suspensions from infant mice inoculated with the adapted virus showed two distinct kinds (large and small) of plaques on rhesus monolayers, but only large plaques appeared in cultures inoculated with a mouse brain suspension of the parent A 9 strain. The large plaque resembling that produced by the parent virus and the small plaques resembling those produced by the adapted strain were found *only* in mouse brain after inocula-

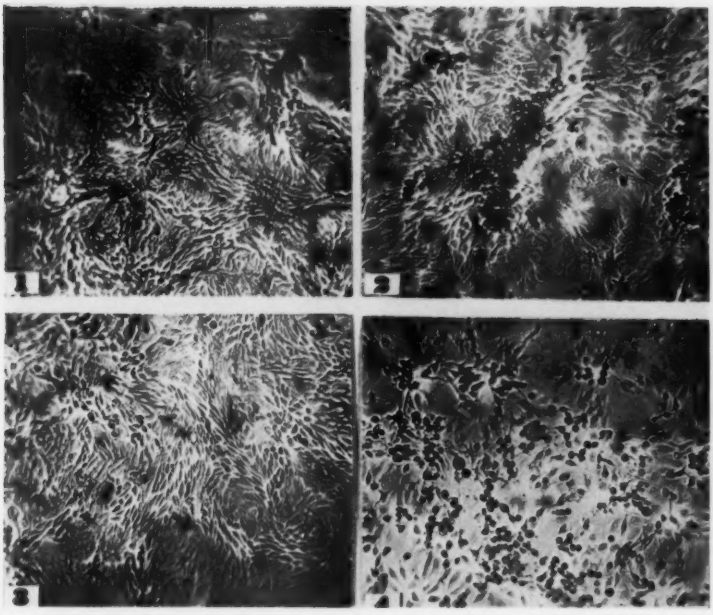

FIG. 2. Cytopathic effect of ECHO-11 (WB) virus in Rh and HK-B cultures.
1. A rhesus control culture.
2. A rhesus culture inoculated with ECHO-11 (WB) virus 48 hrs. after infection showing small foci of degeneration.
3. An infant kidney culture control.
4. An infant kidney culture inoculated with ECHO-11 (WB) virus 8 hrs. after infection showing extensive cellular destruction.

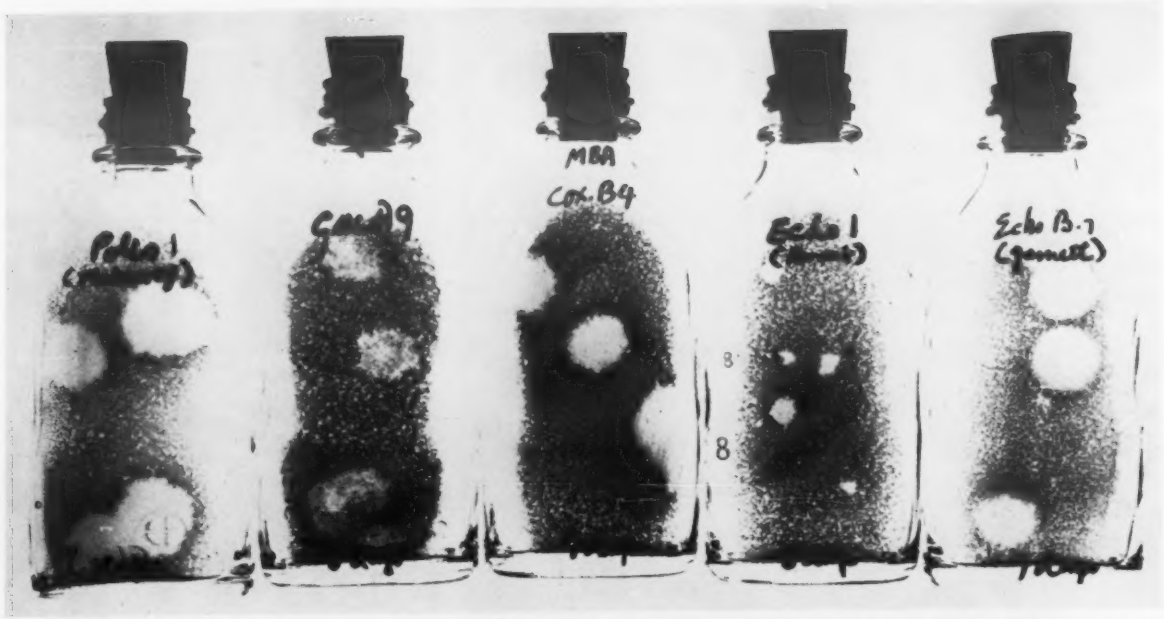

FIG. 3. Plaque patterns of enteroviruses on rhesus cultures. Bottles from left to right: 1. Poliovirus type 1 (Mahoney strain); 2. Coxsackie virus A 9 (Grigg strain); 3. Coxsackie virus B 4 (Powers strain); 4. ECHO virus type 1 (Farouk strain); 5. ECHO virus type 7 (Garnett strain).

FIG. 4. Plaques of attenuated and virulent strains of poliovirus on human kidney cell cultures four days after seeding. Bottles from left to right: 1. Poliovirus type 2 (MEF 1) Virulent; 2. Poliovirus type 2 (YSK) Attenuated; 3. Poliovirus type 3 (Saukett) Virulent; 4. Poliovirus type 3 (Leon) Attenuated.

tion with the *adapted* virus. Viruses recovered from both plaque types were neutralized by Coxsackie A 9 antiserum.

Both the large and small plaques were picked and grown in rhesus tube cultures. The results of further plating of these plaques indicate that viruses from large plaques produced mostly large sized plaques. However, large plaques emerged from small plaque viruses. In general, virus titers in patas cultures were low for large plaques and high for small plaques, resembling those markers for the parent and the adapted strains, respectively.

TABLE 3. CHANGE IN PLAQUE SIZE ON SERIAL PASSAGES IN IRRADIATED PATAS CULTURES

	Plaque type (per cent)		
No. pass. in irrad. patas	*Large (10 mm.)*	*Medium (5-10 mm.)*	*Small (5 mm.)*
0*	100	0	0
1	100	0	0
2	88.4	2.3	9.3
3	26.6	33.3	40.0
4	12.5	12.5	75.0
5	0	25.0	75.0
6	0	0	100.0
10	0	0	100.0
14	0	0	100.0

* Original parent virus.

Growth rate and plaque size of the adapted virus in rhesus and patas cultures

Sets of rhesus and patas cultures were each inoculated with the adapted virus at limiting dilutions and harvested at two-hour intervals. The results are shown in Table 4. In rhesus cultures virus first appeared in four hours and increased in titer up to ten hours. Only the large plaque type virus was observed during this period (first growth cycle). However, small plaques (18 per cent) were obtained in the 24-hour sample, indicating a delayed growth rate of the small plaque-type virus. In patas cultures the virus was first detected after eight hours, and the size of the plaques was small. Approximately four per cent of the virus population formed plaques of medium size in a sample harvested at 24 hours from patas cultures. The growth rate of the small plaque-type virus again was shown to be slower than the large plaque type.

The origin and frequency of this reversion are summarized in Figure 5. This diagramatic scheme illustrates the nature of the experiments conducted to study plaque variation with Coxsackie A 9 virus. All plaques were measured on rhesus monolayers seven days after seeding. The numbers in the small, medium, and large circles give the percentage of the small, medium, and large plaque types in the populations tested.
These data indicate generally that:

TABLE 4. GROWTH RATE AND PLAQUE SIZE OF ADAPTED VIRUS IN RHESUS AND PATAS CULTURES

	Cell culture											
	Rhesus						*Patas*					
Hours after infection	2	4	6	8	10	24	2	4	6	8	10	24
Virus titer log PFU(Rh)/ml.	0	2.0	3.6	3.5	1.0	4.1	0	0	0	2.2	2.9	4.0
Plaque size*	L	L	L	L	L	L&S	S	S	S	S	S	M&S

* L = Large (10 mm.).
M = Medium (5-10 mm.).
S = Small (5 mm.).

(i) After 14 passages of Coxsackie A 9 virus in irradiated patas cultures, the adapted virus produced smaller plaques on rhesus monolayers than the parent strain.

(ii) When a limiting dilution (10 PFU) or a single plaque was transferred to a rhesus culture, only large plaques were evident in harvests at eight hours, that is, at a single growth cycle.

(iii) A shift toward the large plaque size resulting in a mixed population was noted when the adapted virus was passed through newborn mice. Similar results were also obtained in rhesus cultures after multiple growth cycles either in a fluid medium or under an agar overlay.

(iv) One further passage of this mixed population in rhesus cultures yielded a total reversion to the large plaque type. In contrast, three passages in non-irradiated patas cultures produced the same high percentage of small plaque types, and one further mouse passage resulted in only a partial increase in the proportion of the parent plaque type.

It is generally recognized that most variation in virus properties is due to mutation and selective outgrowth of the variant rather than to any direct influence of the environment on the multiplication of virus population.

However, in our study the unusually high rate of reversion in plaque types as well as in host cell range during a single passage in either rhesus cultures or infant mice and the apparently complete reversion when the harvest was made in rhesus cultures after a single growth cycle suggested that the host cell may exert a direct influence on the variation observed in Coxsackie A 9 virus.

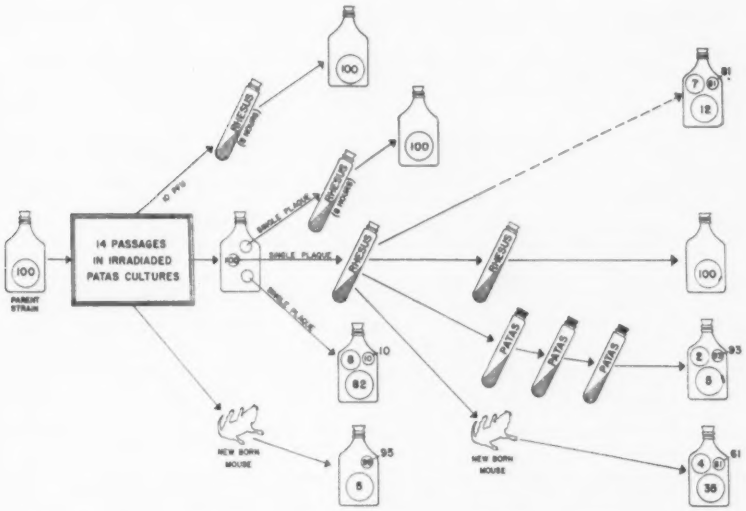

FIG. 5. A summary on plaque variation of Coxsackie A 9 virus. The numbers in the small, medium, and large circles give the percentage of the small, medium, and large plaques in the populations tested.

CONCLUSION

In this symposium we have attempted to review some trends in the application of primary cell cultures in the study of a variety of animal viruses. Cell line cultures of cancerous origin were not utilized in these studies due to the lack of or change in specificity to certain viral infections. To demonstrate such specificity, results may be achieved only when the cultures are derived directly from a specific host. Virologists for the next decade at least will have to continue to be concerned with developing newer methods for the recognition and classification, as well as for the biological and genetic studies of these viruses. We believe that as these techniques become more quantitative, they will contribute substantially to

the solution of the nature and behavior of these viruses and to the effective and efficient control of disease.

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PYELONEPHRITIS. I. OBSERVATIONS ON THE COURSE OF CHRONIC NON-OBSTRUCTED ENTEROCOCCAL INFECTION IN THE KIDNEY OF THE RAT‡

Studies of the pathogenesis of experimental pyelonephritis have been limited because of lack of a suitable model. Although several types of renal and extra-renal manipulations have been shown to predispose the kidneys of experimental animals to pyelonephritis, the investigator is frequently unable to distinguish between the effects of the predisposing injury and the induced infection. The few micro-organisms capable of initiating pyelonephritis in the "non-manipulated" kidney usually cause rapid death of the experimental animal or the renal lesion becomes bacteriologically sterile and the disease does not progress. The present data describes the course of chronic pyelonephritis produced by the intravenous injection of enterococci into the normal rat.

MATERIALS AND METHODS

Animals. White, male, Wistar strain rats weighing 100-200 grams were used.

Bacterial strain. The culture employed was a strain of *Str. faecalis* originally isolated from the urine of a patient with pyelonephritis. The organism was a gram-positive coccus which grew in 6.5 per cent sodium chloride broth, produced acid reaction in mannitol, sorbitol, and glycerol media, did not liquify gelatin, was catalase negative, and did not hemolyze human red blood cells. Before using it in the experiments to be described, the organism was "passed" through a series of normal rats. One week after intravenous inoculation, the kidneys were removed, homogenized and cultured, and the bacteria obtained were then injected into another animal. After three such animal

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passages, the organism was grown in beef heart infusion broth for six hours; 5 ml. portions then being frozen and stored at —20° C. Each week one tube was thawed and incubated for 24 hours at 37° C., then "passed" once more through a rat. The culture recovered, after proper identification, was used for all experiments during the next week.

During the course of experiments the organisms were identified by colony characteristics, Gram-stain, ability to grow in 6.5 per cent sodium chloride broth, and absence of catalase activity.

Injection of organisms. The tail vein was employed for intravenous injection. The inoculum consisted of 1.0 ml. of an 18 to 24 hour beef heart infusion culture which had been incubated at 37° C. Serial ten-fold dilutions of the culture were made in 0.85 per cent sodium chloride solution and aliquots were incubated in agar pour plates to enumerate each inoculum.

Removal of organs for enumeration of bacterial content. Under pentobarbital anesthesia, the abdomen and thorax were opened so that the organ to be examined could be removed and placed in a glass homogenizer. In the case of the kidney or spleen, the entire organ was utilized, whereas with liver, approximately 1 gm. of tissue was removed. The tissue was ground in 9 ml. of 0.85 per cent sodium chloride solution until a smooth suspension was obtained. This represented the 10^{-1} dilution, subsequent ten-fold dilutions being made in the saline solution. Agar pour plates were made from several of these dilutions, depending on the expected number of micro-organisms. After incubation for 48 hours, colony counts were made; the one containing between 30 and 300 colonies being taken as most nearly representative. When the plate containing the greatest dilution contained too many colonies to be counted the number was recorded as greater than 1,000.

Urine cultures. Urine was obtained by needle aspiration of the bladder at the time of sacrifice. The specimens were diluted with 0.85 per cent sodium chloride solution and incorporated in an agar pour plate, for determination of the bacterial content.

Blood cultures. Blood was obtained directly from the heart and 1 ml. was introduced into 10 ml. of beef heart infusion broth. After 48 hours incubation at 37° C. this culture was streaked on blood agar and the resultant bacterial growth studied for the presence of *Str. faecalis.*

Histological studies. At the time of removal of the organs for bacteriological study, pieces of tissue were obtained for histological examination. One-half of spleen, the heart, urinary bladder and a wedge of hepatic tissue were placed in 10 per cent neutral formalin. In the case of the kidney, either the whole organ or one-half was obtained. After proper fixation, the tissues were stained with hematoxylin and eosin.

EXPERIMENTAL

Course of enterococcal infection in normal animals. In order to study the course of infection in normal animals, groups of four to ten rats were sacrificed at intervals of one hour to 57 weeks after the intravenous injection of 400 to 450 million micro-organisms. In general, this size inoculum was well tolerated. None of the animals died in the immediate post-injection period. The occasional death encountered later in the study would be ex-

pected in an animal colony of this size and was not attributed to the infection or its sequelae. The results of bacteriological examination of the kidneys, spleen, liver, urine, and blood of 156 animals are presented in Figure 1.

The course of the infectious process was characteristic for each tissue and there was little individual variation from animal to animal. The findings indicate that larger proportions of the intravenous inoculum were

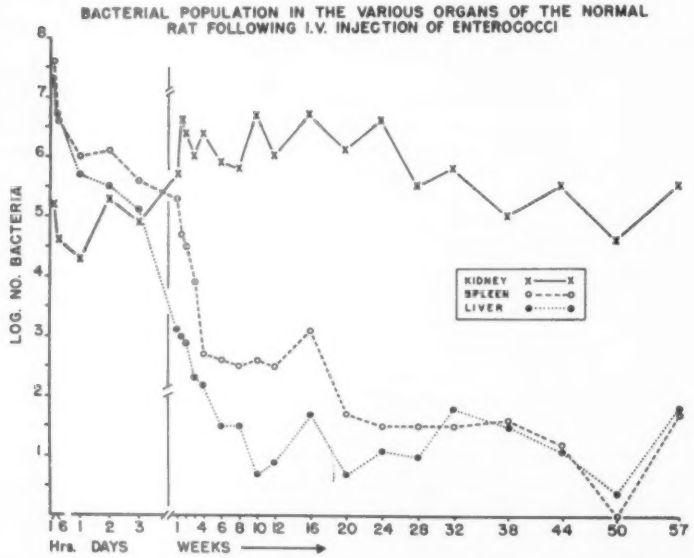

FIG. 1. The bacterial population in the various organs of the normal rat following the intravenous injection of enterococci. The points plotted represent the averages of the logarithms of the numbers of organisms cultured from the whole kidney or spleen, or from 1 gm. of liver. (Portions of these data were published in Medicine, 1960, *39*, 3.)

arrested in the liver and spleen; the kidneys participated to a smaller degree in the removal of bacteria from the circulation. In the liver and spleen, destruction of the microbial population occurred at a relatively constant rate during the first four weeks. Thereafter, reduction in number of bacteria was more gradual. The first instances of negative cultures were noted at six weeks in the liver, and 20 weeks in the spleen. At subsequent times, failure to isolate bacteria from the liver and spleen occurred in occasional animals. In contrast, there was little evidence for the killing of bacteria by

the kidney. The micro-organisms in this organ multiplied, attained a maximum population at 10 days and thereafter persisted in relatively constant numbers throughout the period of observation. In 11 animals sacrificed between one hour and 24 weeks after injection, no evidence of pyelonephritis was found in one of the kidneys examined despite the occurrence of

TABLE 1. OCCURRENCE OF BACTERIURIA FOLLOWING THE INTRAVENOUS INJECTION OF ENTEROCOCCI IN THE RAT

	Log nos. of bacteria	
*Time days**	*Kidney*	*Urine (ml.)*
1	3.6	0†
	3.6	0
	4.6	0
	4.6	0
2	3.6	0
	5.6	0
	3.3	0
	5.9	0
	3.5	0
3	6.5	5.4
	6.6	4.8
	5.0	4.4
	6.7	5.0
5	6.5	4.2
	5.9	6.9
	7.4	4.8
	0	0

* This represents the interval between injection of bacteria and time of sacrifice.
† In these experiments, 0 means that no organisms were cultured from 1 ml. of urine.

infection in the contralateral organ. Whether this represented a temporary vascular phenomenon which effectively prevented the access of bacteria to the kidney or a unilateral increased resistance to infection was not determined. The data obtained from these kidneys were not included in Figure 1. At 28 weeks, one animal was examined in which cultures of one kidney were negative despite the presence of macroscopic and microscopic evidence of chronic inflammation and scarring. The other kidney presented bacteriological and pathological findings of pyelonephritis. This observation of unilateral "burning out" of renal infection was noted in four of 27 animals examined between 32 and 57 weeks.

Urine cultures were obtained during the course of these experiments. These data indicated a failure to recover bacteria from the urine during the first two days after intravenous injection. The next group of urine cultures as positive but since these were obtained on the seventh day, it was not possible to determine the exact time of appearance of bacteriuria. Accordingly, the experiments were repeated and animals were sacrificed one, two, three and five days after intravenous injection. Cultures were made of urine and one kidney. The results are presented in Table 1. Similar findings were noted in that none of the nine rats examined at one and two days had evidence of bacteriuria. However, enterococci were recovered from the urine of all but one of the animals studied on the third and fifth days. This one failure to isolate enterococci from the urine in an animal without bacteriological evidence of renal infection suggested a faulty intravenous injection. It should be mentioned that the appearance of bacteriuria coincided with the earliest demonstrable histological lesions in the renal medulla. This indicates that the kidney does not act as a "filter" for bacteria, but rather that infection with associated tissue destruction and rupture of micro-abscesses into the tubular collecting system is necessary for the occurrence of bacteriuria. It is interesting to note that continued observations during the course of these experiments demonstrated a consistently high level of bacteriuria. In general, more than 100,000 enterococcus colonies per ml. of urine were found in animals studied between one and 57 weeks after injection. In no instance was a negative urine culture obtained in an animal which had evidence of pyelonephritis.

Blood cultures were obtained in 15 rats one to seven days after intravenous inoculation and in all of these enterococci were found. None of the four animals examined at ten days had evidence of continuing bacteremia. Blood cultures were negative in the 23 random examinations performed between 3 and 57 weeks after infection.

Comparative numbers of organisms in the normal renal cortex and medulla at different intervals after intravenous injection of enterococci. Observations were now made on the quantitative distribution of bacteria in the various parts of the kidney during the course of infection. In order to separate these tissues the kidney was bisected in a transverse plane and pieces of cortex and medulla removed. These were weighed separately on a Mettler Analytical Balance, Type B6 (Mettler Instrument Corp., Highstown, New Jersey) and homogenized in 0.85 per cent sodium chloride solution. The weights of the tissue ranged between 100 to 125 mgm. and 15 to 25 mgm., for the cortex and medulla respectively. The results of these

studies in 82 animals are presented in Figure 2 and are expressed on the basis of number of bacteria per 100 mgm. of tissue.

Although a larger number of enterococci initially localized in the renal cortex, this part of the kidney appeared effectively able to temporarily "handle" this inoculum as manifested by a decrease in bacterial content during the first two days. The resistance of the medulla, however, was not as efficient. Microbial multiplication occurred and a high population was

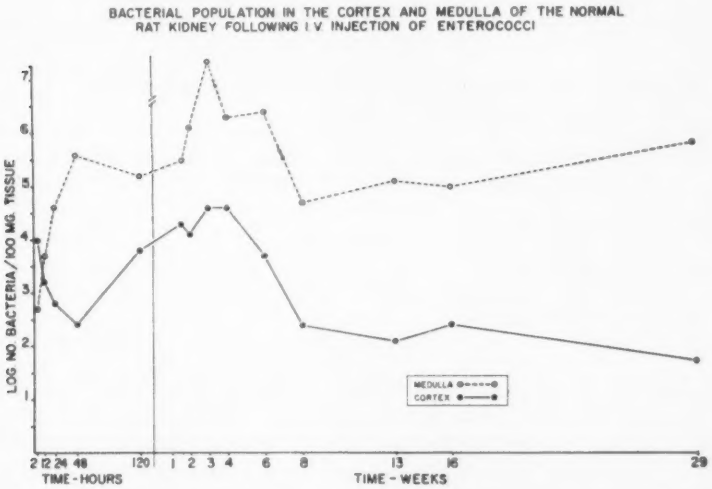

FIG. 2. The bacterial population in the cortex and medulla of the normal rat kidney following the intravenous injection of enterococci. The points plotted represent the average of the logarithms of the numbers of organisms cultured. (Portions of these data were published in *Medicine*, 1960, *39*, 3.)

noted at two days. After this time the medullary bacterial count remained greater than that of the cortex throughout the period of observation. It should be noted that the earliest histological lesions were observed in the medulla two to three days after intravenous injection. These findings occurred during the period of rapid bacterial multiplication in this area. Abscesses appeared in the cortex between the third and fifth days, at a time when there was a secondary rise in the number of enterococci in this part of the kidney.

It is not apparent from these data whether the increase noted in the cortex represents a proliferation of micro-organisms originally lodged in this area or indicates a spread of infection from medulla to cortex.

In these experiments cultures of both cortex and medulla from the same kidney were negative in ten animals examined between five days and 29 weeks after injection. Whether these represent instances of incomplete sampling or "unilateral pyelonephritis" is not known. The data obtained from these ten kidneys were not included in Figure 2.

Determination of the number of organisms required to cause infection in the normal kidney. Groups of rats were given 1.0 ml. injections of serial ten-fold dilutions of enterococcus culture and one week later the kidneys were homogenized and cultured quantitatively. The results are presented

TABLE 2. RELATION OF NUMBER OF ENTEROCOCCI INJECTED INTRAVENOUSLY TO INCIDENCE OF PYELONEPHRITIS IN THE RAT

Dilution (and approximate number of organisms injected)	*Number of animals*	*Number in which pyelonephritis occurred*
Undiluted (400-450 million)	14	14
10^{-1} dilution (40-45 million)	8	4
10^{-2} dilution (4-4.5 million)	8	1
10^{-3} dilution (400-450 thousand)	12	1
10^{-4} dilution (40-45 thousand)	8	1

in Table 2. An intravenous inoculum of 400 to 450 million organisms caused infection in all 14 of the animals studied. When the number of bacteria injected was reduced the proportion of animals infected diminished. At dilutions of 10^{-3} or less, only an occasional rat developed pyelonephritis.

The results obtained in these experiments are of interest when examined with the data shown in Figure 1. An intravenous injection of 400 to 450 million organisms resulted in a bacterial population of approximately 100,000 in the kidney at one hour. If the proportion of injected bacteria lodging in the kidney does not change when the inoculum is reduced, then an injection of 1 ml. of the 10^{-3} dilution or less would result in fewer than 100 micro-organisms being deposited in the kidney. This type of data permits the establishment of an estimated tissue population necessary to produce infection. Thus, in animals receiving 40 to 45 million organisms (1 ml. of 10^{-1} dilution), the infecting tissue population which caused pyelonephritis in 50 per cent of animals is calculated to be approximately 10,000 viable units.

Ability of various Group D Streptococci to produce pyelonephritis in the rat. Observations were made on the occurrence of renal infection following the inoculation of various Group D streptococci into normal rats.

Since some of the strains to be studied had been maintained *in vitro* for many years, it was decided to "pass" the cultures one time prior to testing as a check for pathogenicity. To insure recovery of bacteria an 18- to 24-hour culture of the organism to be tested was injected into a rat in which the left ureter had previously been ligated. Twenty-four hours later, the hydronephrotic kidney was removed, homogenized, and cultured. The bacteria isolated, after proper identification, were incubated at 37° C. for 18 to 24 hours in beef heart infusion broth. Aliquots of these cultures were

TABLE 3. INCIDENCE OF PYELONEPHRITIS FOLLOWING THE INTRAVENOUS INJECTION OF GROUP D STREPTOCOCCI INTO THE RAT

Species	*Source*	*Number of animals*	*Number infected*
Str. faecalis	ATCC 10541	10	9
Str. faecalis	pyelo. urine	9	9
Str. faecalis	pyelo. urine	10	9
Str. faecalis var. *zymogenes*	ATCC 6055	10	8
Str. faecalis var. *liquefaciens*	ATCC 13398	5	5
Str. durans	ATCC 6056	10	0

injected intravenously into groups of normal rats. The number of bacteria injected ranged between 230 millions and 635 millions in a 1 ml. volume. One week later, the animals were sacrificed and the kidneys examined for pathological and bacteriological evidence of infection. The results of these experiments are presented in Table 3. Three strains of *Str. faecalis,* two of which were recently isolated from the urine of patients with pyelonephritis, produced renal infection in the majority of animals tested. Similar results were obtained with strains of *Str. faecalis var. zymogenes and Str. faecalis var. liquefaciens.* The single culture of *Str. durans* tested failed to produce renal infection in any of the animals injected.

Pathology of lesions produced by Str. faecalis in the rat. Macroscopic and microscopic observations were made of the organs of the animals at the time of sacrifice. The liver, spleen, kidneys, urinary bladder, and heart were examined, then fixed in 10 per cent neutral formalin and sections stained with hematoxylin and eosin. In the case of the heart, this organ was dissected under a fixed hand lens; care being taken to note alterations of the valves.

The only pathological changes observed in the liver were small perivascular collections of mononuclear cells first seen two to three days after infection. This process subsided by the seventh to tenth day without apparent residua. In the spleen, moderate follicular hyperplasia was noted during this period but tended to subside by the tenth day. An occasional animal examined after this period had evidence of splenic hyperplasia but these changes were confined to rats with acute exacerbations of chronic renal infection. The heart was examined in approximately two-thirds of the animals studied in these experiments. In no instance was endocarditis, pericarditis, or myocarditis noted.

The major pathological alterations were confined to the kidneys. Macroscopically, the earliest lesions noted were discrete or confluent cortical abscesses, measuring 1-3 mm. in diameter. When the kidney was cut, these cortical lesions could be traced to their origin in the medulla by the appearance of radial streaking of purulent material in the medullary segments adjacent to the involved cortex. These were present in approximately one-half of the animals studied on the fifth day. Thereafter, the frequency of occurrence of renal lesions increased with time so that almost all of the kidneys examined after the sixth week had anatomical evidence of infection. Abscesses persisted in some animals up to four weeks after inoculation, although in the majority of instances, evidence of healing became apparent by the fourteenth day as manifested by diminution in the amount of purulent material and beginning contraction of the involved tissue. At six weeks evidences of scar formation, depressed and irregular in shape, with flat "U-shaped" bases, were seen in most animals (Fig. 3). As the experiments progressed, occasional instances were noted in which acute abscesses were found adjacent to areas of scar tissue. These corresponded with some increase in the bacterial population in the kidney and were interpreted as representing acute exacerbations of chronic pyelonephritis.

Although the earliest macroscopic lesions were noted five days after injection, microscopic changes occurred before this time. At two days, small, focal collections of leukocytes were seen in the interstitium of the renal medulla and papilla. These increased in size during the next two or three days and in some instances ruptured into the tubular collecting system (Fig. 4). During the first three to four days after infection, no lesions were noted in the cortex. Thereafter, interstitial abscesses were seen. Throughout this acute period, the glomeruli remained remarkably clear of evidence of infection. After the fourteenth day the abscesses began to resolve as manifested by the presence of lymphocytic infiltration, diminu-

tion of polymorphonuclear leukocytes and the occurrence of macrophages. Fibroblasts were seen between the third and fourth weeks and thereafter healing and scar formation was noted. As this process progressed, areas of dilated tubules with flattened lining epithelial cells and occasionally filled with pink staining amorphous material were observed between, and adjacent to, the scars (Fig. 5). In the animals studied between 50 and 57 weeks, an occasional instance of beginning periglomerular fibrosis was seen. Blood vessel changes during the course of the infection were surprisingly minimal. In areas of acute inflammation, there was limited involvement of the vessel walls as manifested by edema and leucocytic infiltration. These findings disappeared as the pyelonephritic lesions became sub-acute and chronic. In the later stages, careful examinations failed to reveal any evidence of arteriolarsclerosis.

Despite the occurrence of marked changes in the kidney and persistent bacteriuria, the alterations in the urinary bladder were minimal. In occasional animals, submucosal focal collections of lymphocytes were seen. In no instance was evidence noted of acute cystitis or disruption of the epithelial lining of the bladder.

DISCUSSION

The data presented here indicate that enterococcal pyelonephritis in the rat is a chronic, progressive disease with little tendency for the infection to "burn-out" and the lesions to become bacteriologically sterile. This is in contrast to the sequence of events in the liver and spleen where, although large numbers of micro-organisms initially lodged, these organs were effectively able to gradually destroy the majority of bacteria and anatomical evidence of infection was not noted. In this regard, the behavior of enterococci in the various tissues may be likened to the course of staphylococcal infection in the mouse. Smith and Dubos[8] noted that several strains of staphylococci were eliminated by the liver and spleen of animals examined 21 to 36 days after injection while the microbial population in the kidneys persisted at high levels during this time. This difference in organ susceptibility might be expected if one compares the lack of cellular defense mechanisms characteristic of the kidney with the abundant reticuloendothelial system of the liver and spleen.

Comparative bacteriological studies of the cortex and medulla after the intravenous injection of bacteria demonstrated the importance of this type of approach in order to better understand the host-parasite relationship at specific tissue levels. These observations indicated that the cortex is more resistant to enterococcal infection than is the medulla. Histological observa-

tions confirmed this impression; the earliest micro-abscesses were seen in the medulla, at the time of active bacterial multiplication in this area. Similar lesions appeared in the cortex but at a later period and these also were noted during an interval of rising bacterial content in this tissue. Freedman and Beeson[4] described an increased susceptibility of the medulla to infection as compared to cortex when bacteria were injected directly into these tissues. The altered resistance of the medulla was attributed to the mechanical effects of injecting even small volumes of inocula into this area; this procedure produced tubular obstruction and foci of intrarenal hydronephrosis. Our data, obtained in studies of enterococcal infection in the rat, indicate that the medulla is more susceptible to infection even in the absence of obstruction to urine flow.

The behavioral differences of the various parts of the kidney in regard to infection are difficult to explain. These are apparently not due to an increased localization or "trapping" of bacteria in the medulla. Such a mechanism has been suggested by Lovell (as cited by Gorrill[5]) in *Corynebacterium renale* infection in mice. In these animals, cold acetone extracts of *C. renale* produced cortical ischemia and thus effectively could shunt the injected organisms into the medulla and might account for the medullary distribution of the lesions noted histologically. It is unlikely that similar mechanisms were operative in the enterococcal infection described since the numbers of enterococci in the medulla was less than that of the cortex in animals examined two hours after injection.

Since *C. renale* is a powerful urease producer, it has been suggested that this would favor its multiplication in the urea-rich area of the kidney. Confirmation of this was obtained by Lister[7] who described a lower incidence of renal infection in mice given injections of a urease-negative mutant of *C. renale* when compared with the occurrence of pyelonephritis following the inoculation of the urease producing strain. This mechanism was not operative in the present experiments since the strain of *Str. faecalis* used failed to produce urease when tested in Christensen's Urea medium.

This concept of a specific tissue supplied substrate which favors bacterial growth can be further extended as an alternative explanation of corticomedullary differences in susceptibility to enterococcal infection. The ability of enterococci to grow in relatively high sodium chloride concentrations *in vitro* is well known. It has been established that a gradient of sodium chloride concentration exists in the kidney with the relatively higher concentrations found in the papillae decreasing as the cortex is approached. Whether or not these solute differences are important has not been determined.

FIG. 3. Rat kidney 24 weeks after enterococcal infection. Note the large irregular shaped scar at the upper pole.

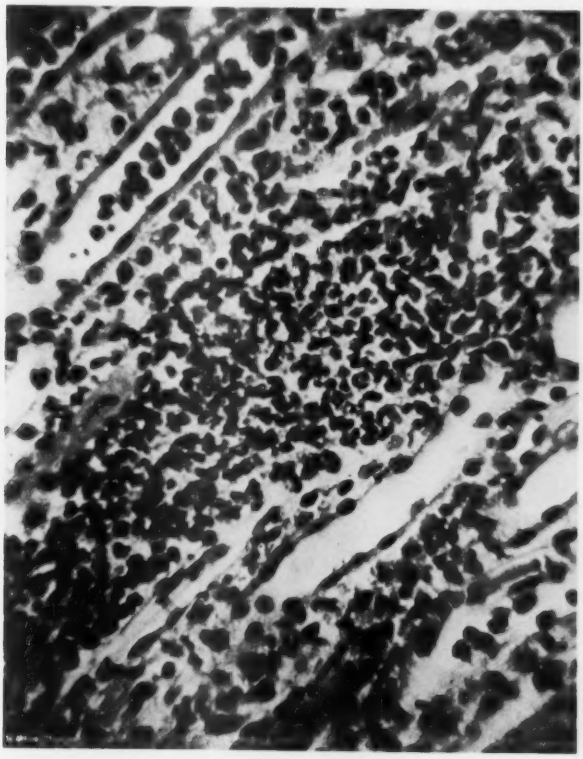

FIG. 4. Medulla of rat kidney two days after enterococcal infection. Note interstitial abscess which has ruptured into surrounding tubules. (x300).

FIG. 5. Rat kidney one year after enterococcal infection. Note the destruction of tissue, lymphocytic infiltration and dilated tubules containing pink-staining colloid material. (x100).

The renal localization of staphylococci has been attributed to the ability of this organism to coagulate plasma and thus effectively "wall-off" the multiplying bacteria from the cellular and humoral defences of the host.[8] It has been shown, however, that kidney infection was produced in the mouse by strains of staphylococci which were not able to coagulate mouse plasma.[8] Attempts to demonstrate coagulase production by the strain of enterococcus described, using human and rat plasma, were unsuccessful.

Beeson and Rowley[1] have recently presented data which may relate to this problem. In an excellent study of the susceptibility of the kidney to coliform bacterial infection, these investigators noted that kidney tissue, unlike that of other organs, interferes with the ability of normal serum to destroy these organisms. This effect was attributed to the anticomplementary activity of renal tissue; the fourth component of complement being primarily involved. This component is characterized by susceptibility to injury by ammonia and the studies described were consistent with the hypothesis that the mechanism of anticomplementary effect was associated with ammonia formation. This basic contribution suggests the existence of a biochemical factor which might predispose the kidney to infection and offers a new approach to the understanding of the pathogenesis of pyelonephritis. It is not known whether such a mechanism is involved in the enterococcal infection described, but data are available which indicate that the ammonia content of the medulla is greater than that of the cortex and speculation might be made about the importance of these differences in the relative susceptibility of the cortex and medulla to infection.

The quantitative aspects of the inoculum size and the frequency of infection are of interest in that there appears to be a roughly linear relationship between the number of bacteria injected and the incidence of pyelonephritis. Similar findings were noted in staphylococcal infection of the mouse kidney by Gorrill[6] who described a linear proportionality between the number of bacteria injected and the microbial population of the individual kidney. This type of data permitted Gorrill to estimate the kidney infecting dose (KID_{50} staphylococcus) and thus enabled him to design experiments concerned with the quantitative aspects of the host-parasite relationship. Although observations of the dose-renal implant ratios were not made in the present experiments, the assumption that these are in linear relationship may be made in light of Gorrill's work. On this basis it was calculated that approximately 10,000 viable units of enterococci must lodge in the rat kidney to initiate infection in half of the animals tested (KID_{50} enterococcus).

The failure to demonstrate bacteriuria in the first two days after intravenous inoculation despite the presence of large numbers of micro-organ-

isms in the kidney and the appearance of enterococci in the urine coincident with the appearance of micro-abscesses supports the hypothesis that the kidney does not function as a filter of bacteria.[8] Tissue damage must occur in order to permit bacteria to escape into the tubular collecting system and appear in the bladder urine. The lack of evident cystitis in animals with chronic pyelonephritis and significant bacteriuria suggests a remarkable protective ability of the intact bladder mucosa.

The inability of the strain of *Str. durans* to produce renal infection may offer a tool with which individual parasite factors affecting the renal host-resistance may be studied. This organism differs from *Str. faecalis* in its failure to decolorize litmus milk, lower saccharolytic activity, sensitivity to tellurite and lower tyrosine decarboxylase activity.[9] The role these and other differences play in the pathogenesis of infection is at present under investigation.

The chronicity and progressive nature of the enterococcal pyelonephritis described in these experiments provides a model in which observations may be made of the course and natural history of this disease. It may now be possible to study the association of hypertension and kidney infection, the pathogenesis of the anemia of infection and/or uremia, and the quantitative aspects of the host-parasite-drug relationship as it relates to this micro-organism.

SUMMARY

A model of chronic, progressive enterococcal pyelonephritis in the rat has been described. This infection is characterized by the persistence of bacteria in relatively constant numbers during the evolution of the disease from the acute to chronic stage. Quantitative bacteriological data, obtained during observation of this infection for more than one year, were presented.

A comparatively large number of bacteria was required to cause infection. It was estimated that the kidney infecting dose necessary to produce pyelonephritis in 50 per cent of animals tested (KID_{50}) was 10,000 viable enterococci.

Quantitative observations were made on the microbial populations in the cortex and medulla at various intervals after intravenous injection of bacteria and evidence was presented which indicated that the medulla was the part of the kidney most susceptible to infection. It was also in this area that the earliest histological evidences of infection were noted.

Bacteria were not found in the urine of animals examined one and two days after inoculation but were cultured on the third day. This coincided

with the appearance of the earliest microscopic abscesses in the medulla. The absence of bacteriuria in the presence of large number of organisms in the kidney confirms the observation that the kidney does not act as a "filter" for bacteria but rather that infection with associated tissue destruction must be present for micro-organisms to gain access to the urine.

Several strains of *Str. faecalis, Str. faecalis var. zymogenes,* and *Str. faecalis var. liquifaciens* produced renal infection in the majority of rats injected. A single culture of *Str. durans* failed to cause infection in any of the animals tested.

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STIMULATION OF ALDOSTERONE SECRETION BY ANGIOTENSIN II

A Preliminary Report†

INTRODUCTION

The role of the renin-angiotensin system in renal hypertension has been investigated for many years but the relationship of the renin-angiotensin system to adrenocortical function has received less attention. In 1951, Deane and Masson,[4] using the rat as an experimental animal, reported that encapsulation of the kidney, a procedure which results in hypertension presumably through release of renin, and injections of crude renin preparations both produced enlargement of the zona glomerulosa of the adrenal gland, the site of aldosterone formation. Hartroft and co-workers[6, 16] noted that salt loading in the rat decreased both the granulations of the juxtaglomerular apparatus (the probable site of renin formation) and the content of renin in the kidney, while sodium deprivation increased the granulations and the content of renin. An excellent review by Tobian[19] has summarized the data concerning the possible role of the renin-angiotensin system in electrolyte metabolism and hypertension. Recently prolonged infusions of synthetic angiotensin into man have been reported as producing a pressor response and an increase in aldosterone excretion and secretion.[5, 10] It is a possibility that the aldosterone effect was an indirect one through angiotensin stimulating ACTH release from the pituitary. In fact, Genest[5] found that the angiotensin infusion also increased urinary tetrahydrocortisol excretion. Experiments conducted independently by Davis and co-workers[3] and the authors[12] have implicated the kidney and the renin-angiotensin system in the increased aldosterone secretion that occurs fol-

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lowing hemorrhage in the hypophysectomized dog. The present report concerns a preliminary study of the effect of angiotensin II on adrenocortical secretion in the hypophysectomized, nephrectomized dog.

PROCEDURE AND METHOD

Angiotensin II Experiments. Mongrel male dogs weighing about 12 kilograms underwent a left nephrectomy one to four weeks prior to the experimental day. On the experimental day, each dog was anesthetized with Nembutal, acutely hypophysectomized by the transbuccal route, following which the right kidney was removed and the right lumboadrenal vein cannulated by the method of Nelson and Hume.[14] The femoral artery was cannulated for continuous blood pressure recording using a Grass Model 5 Polygraph and a Statham strain gauge, and the femoral vein for administration of blood or angiotensin II. During the control period, two 10-15 ml. samples of adrenal vein blood were collected at 20-minute intervals. The dog was then hemorrhaged 15 ml./kg. body weight and three post-hemorrhage samples of adrenal vein blood collected at 20- to 30-minute intervals. Following the last sample, isotonic saline solutions containing 3.3 μg of synthetic angiotensin II* per ml. were infused through a constant infusion pump. Two more adrenal vein blood samples were collected, one during the infusion and the other one-half hour after its completion.

In one experiment (#224), following the last post-hemorrhage sample, 10 μg of angiotensin II in 3 ml. of isotonic saline was administered intravenously over one minute and adrenal vein blood collected for 18 minutes. One-half hour after the angiotensin II injection, 5 milliunits of Upjohn ACTH in 1 ml. of isotonic saline were administered intravenously over one minute and adrenal vein blood was collected for 18 minutes.

ACTH experiments. The effect of graded doses of Upjohn ACTH on adrenocortical function was studied. On the experimental day, Dog #111 was anesthetized with Nembutal, hypophysectomized through the transbuccal route, and the right lumboadrenal vein cannulated. At one-half hour intervals a dose of ACTH (5 mμ. per ml. of saline) was injected intravenously and adrenal vein blood collected from four to fourteen minutes following the injection.

Following a study of the effect of acute respiratory acidosis on adrenocortical secretion of a normal dog (Dog #13) the effect of intravenous administration of 10 and 1000 mμ of Armour ACTH in saline was studied. On the experimental day, the dog was anesthetized with Nembutal, the right lumboadrenal vein cannulated, and a tracheal cannula inserted for regulation of the dogs respirations by a respiratory pump. During the control period, 100 per cent oxygen was administered through the tracheal cannula. The dog was then respired with a mixture of 90 per cent oxygen and 10 per cent carbon dioxide for approximately one and one-half hours. Three samples of adrenal venous blood were collected in the control and the respiratory acidosis periods at one-half hour intervals. Following the respiratory acidosis period, the dog was respired with 100 per cent oxygen. After twenty minutes a "control' sample of adrenal venous

* Synthetic angiotensin II (Ciba-Hypertension II) was obtained through the courtesy of Ciba Pharmaceutical Inc.

blood was collected. Ten milliunits of Armour ACTH in 1 ml. of isotonic saline was then injected intravenously. Nine minutes later, a five-minute collection of adrenal venous blood was begun. Approximately seventeen minutes after the initial injection, 1000 milliunits of Armour ACTH in 1 ml. of isotonic saline was administered intravenously and collection of adrenal venous blood started five minutes later.

In all dogs, immediately after the collection of each sample of adrenal vein blood, an equal volume of dog donor blood was administered to avoid the effect of repeated small hemorrhages. There was no replacement of the blood removed in the large hemorrhage in the hypophysectomized, nephrectomized dogs.

TABLE 1. ANGIOTENSIN EXPERIMENT

Dog #215—12 Kg. Hypophysectomized, Nephrectomized

Time mins.	*B.P.* mm./Hg*	*Adrenal plasma flow** ml./min.*	*17-OH Corticoids† µg./min.*	*Corticosterone µg./min.*	*Aldosterone mµg./min.*
-40	65/25	0.9	0	0.058	19
-10	70/30	0.8	0	0.048	17
0-17	Angiotensin infusion 1.67 µg./min.			Total 28µg.	
6	150/100	0.7	2.2	0.67	44
53	100/60	1.4	0	0.078	17
58-71	Angiotensin infusion 0.42 µg./min.			Total 6µg.	
64	145/100	1.1	0.8	0.27	37
107	105/55	1.5	0	0.055	18
112-123	Angiotensin infusion 0.17 µg./min.			Total 2µg.	
118	120/70	1.3	0	0.056	15
160		1.0	0	0.067	26

* Blood pressure.
** Adrenal plasma flow was calculated from the adrenal blood flow and hematocrit.
† 17-OH corticoids is used for 17-hydroxycorticoids.

Corticosterone and aldosterone were measured in adrenal vein plasma by the isotope derivative method of Kliman and Peterson, 17-hydroxycorticoids by the Silber-Porter method.[17] Secretory rates were calculated from the concentration and the plasma flow per minute.

RESULTS

In Dog #111 only are the results of the entire experiment shown. The effect of hemorrhage on adrenocortical secretions of the hypophysectomized, nephrectomized dogs will be published elsewhere as part of a larger series of experiments. The possibility that the prior experimental conditions influenced the pattern of response of the adrenal cortex to angiotensin or ACTH cannot be excluded.

Angiotensin II experiments. The infusion of approximately 30μg of Angiotensin II stimulated secretions of 17-hydroxycorticoids, corticosterone, and aldosterone in three of the hemorrhaged, hypophysectomized, nephrectomized dogs (Tables 1, 2, 3). Cessation of the infusion resulted in the return of the secretory levels to the base line. In each dog, a lower dose of Angiotensin II was found which reduced the increment in 17-hydroxycorticoids and corticosterone secretion. However, aldosterone secretion was stimulated to approximately the same extent. In fact, in Dog #223 (Table 3) an infusion of 3μg of Angiotensin II stimulated only aldosterone secretion. In the hemorrhaged, hypophysectomized, nephrectomized Dog #224, a comparison between the effects of a single injection of Angiotensin II and 5 milliunits of ACTH on adrenocortical secretions was made. Angiotensin stimulated all three parameters, 17-hydroxycorticoid, corticosterone and aldosterone secretion, while ACTH stimulated only 17-hydroxycorticoid and ACTH secretion (Table 4).

TABLE 2. ANGIOTENSIN EXPERIMENT

Dog #218—12 Kg. Hypophysectomized, Nephrectomized

Time mins.	*B.P.* mm./Hg*	*Adrenal plasma flow** ml./min.*	*17-OH Corticoids† μg./min.*	*Corticosterone μg./min.*	*Aldosterone mμg./min.*
-40	90/45	0.7	0	0.031	1.9
-10	100/50	0.6	0	0.044	0.7
0-19	Angiotensin infusion 1.67 μg./min.			Total 32μg.	
6	185/125	0.3	2.6	0.81	23
49	100/45		..	....	...
49-78	Angiotensin infusion 0.42 μg./min.			Total 12μg.	
54	150/95	0.2	0.2	0.14	18

* Blood pressure.
** Adrenal plasma flow was calculated from the adrenal blood flow and hematocrit.
† 17-OH corticoids is used for 17-hydroxycorticoids.

Angiotensin II, a potent pressor substance, always produced a rise in blood pressure, but not necessarily to levels abnormal for the dog. Two of the dogs were hypotensive prior to the initial angiotensin infusions, while the blood pressure of Dog #223 had returned to the pre-hemorrhage level of 150/100mm./Hg. In this dog, 3μg of Angiotensin II increased aldosterone secretin to 33 mμg./min. while only raising the blood pressure to 155/95 mm./Hg. Although this was an increase over the blood pressure

of 125/45 recorded immediately prior to the angiotensin infusion, the level is comparable to the "control" blood pressure of 150/100mm./Hg. when the aldosterone secretory rate was only 6mμg./min.

The effect of Angiotensin II upon adrenal blood flow was variable.

ACTH experiments. Commercial ACTH produced a dose response pattern of adrenocortical secretion different from Angiotensin II. Under different experimental conditions, both preparations of ACTH produced simi-

TABLE 3. ANGIOTENSIN EXPERIMENT

Dog #223—11 Kg. Hypophysectomized, Nephrectomized

Time mins.	*B.P.* mm./Hg*	*Adrenal plasma flow** ml./min.*	*17-OH Corticoids† μg./min.*	*Corticosterone μg./min.*	*Aldosterone mμg./min.*
-50	110/60	0.9	0	0.023	3
-20	150/100	0.5	0	0.017	6
0-18	Angiotensin infusion 1.67 μg./min.			Total 30μg.	
6	190/150	0.5	2.1	0.40	30
48	150/75	0.2	0	0.010	15
58-70	Angiotensin infusion 0.42 μg./min.			Total 5μg.	
64	190/145	1.4	3.0	0.57	61
106	100/30	1.3	0.4	0.028	4
110	125/45	..	..	....	..
111-127	Angiotensin infusion 0.167 μg./min.			Total 3μg.	
117	155/95	0.8	0.2	0.010	33

* Blood pressure.
** Adrenal plasma flow was calculated from the adrenal blood flow and hematocrit.
† 17-OH corticoids is used for 17-hydroxycorticoids.

lar results (Tables 4 and 5). The largest dose stimulated both glucocorticoid secretion (17-hydroxycorticoids or corticosterone). There were no changes in arterial blood pressure following ACTH administration.

The volumes of isotonic saline administered were small in all experiments and probably did not influence the results. Moreover, administration of 100 ml. of isotonic saline over one minute to each of three hypophysectomized, nephrectomized dogs by the authors had no significant effect on adrenocortical secretions.

DISCUSSION

Angiotensin is a decapeptide that is formed *in vivo* by the action of renin, an enzyme released from the kidney, on the α^2 globulins of blood. A specific

converting enzyme in plasma converts angiotensin I into the potent pressor octapeptide angiotensin II.[18]

The pattern of adrenocortical response to short term intravenous administration of synthetic angiotensin II depended upon the dose administered. In each of three dogs, a dose of about 30μg. of angiotensin II stimulated both glucocorticoid and aldosterone secretion while a lower dose stimulated primarily aldosterone secretion. The values of glucocorticoid secretion at-

TABLE 4. ANGIOTENSIN AND ACTH EXPERIMENT

Dog ‡224—10 Kg. Hypophysectomized, Nephrectomized

Time mins.	*B.P.* mm./Hg*	*Adrenal plasma flow** ml./min.*	*17-OH Corticoids† μg./min.*	*Corticosterone μg./min.*	*Aldosterone mμg./min.*
-60	125/70	1.1	0	0.067	0
-30	125/55	1.2	0	0.051	0
0	120/55	Angiotensin II 10 μg. over 1 minute			
0-18	151/93‡	1.5	1.4	0.30	17
18	120/65	..	..	....	..
30	125/60	ACTH¶ 5 mμ over 1 minute			
30-48	120/55	1.3	3.2	0.49	3
48	125/60	..	..	....	..
60	125/60	1.4	0.1	0.043	1

* Blood pressure.
** Adrenal plasma flow was calculated from the adrenal blood flow and hematocrit.
† 17-OH corticoids is used for 17-hydroxycorticoids.
‡ Average blood pressure for 18 minutes. Peak blood pressure 190/135 mm./Hg occurred within one minute, lasted for two minutes and gradually declined thereafter.
¶ Lyophilized Upjohn Adrenocorticotropin Hormone.

tained even with the 30μg. dose were still well below the maximum secretory rate. However, the aldosterone secretory rates attained were as high or higher than the secretory rates following hemorrhage in the hypophysectomized dog.[18] It should be kept in mind, however, that the response of the adrenal to short term infusions of angiotensin II may be different from the response to a prolonged infusion. A study by Hilton, *et al.*[7] using the perfused isolated dog adrenal showed that the stimulatory effect of a vasopressor infusion on cortisol secretion was transient and not sustained by a prolonged infusion.

Similarly the pattern of adrenocortical response to intravenous administration of ACTH is dose-dependent. Like angiotensin, the larger doses of ACTH stimulated both glucocorticoid and aldosterone secretion while,

in contrast to angiotensin, the smaller doses stimulated only glucocorticoid secretion. There were smaller doses of ACTH which even produced a higher glucocorticoid secretory rate than the 30μg of angiotensin yet did not stimulate aldosterone secretion. It should be pointed out that the experimental design of the angiotensin and ACTH experiments differed. It is possible that the metabolic state of the adrenal gland influenced the results. This possibility did occur in a previous study by the authors[18] in

TABLE 5. ACTH EXPERIMENTS

Treatment	*Time mins.*	*Adrenal plasma flow* ml./min.*	*17-OH Corticoids** μg./min.*	*Corticosterone μg./min.*	*Aldosterone mμg./min.*
		Dog #111—12 Kg. Hypophysectomized			
Control	—6	1.7	1.2	0.11	15
1 mμ.† ACTH‡	0	1.4	0.6	0.19	10
5 mμ. ACTH	30	1.4	2.5	0.81	8
10 mμ. ACTH	60	1.3	5.9	1.74	7
25 mμ. ACTH	90	1.2	7.1	2.17	10
2 mμ. ACTH	120	1.0	0.6	0.31	11
0	150	1.3	0.5	0.13	11
50 mμ. ACTH	180	1.4	9.3	3.14	33
		Dog #13 "Normal"			
Control	—8	0.9	..	1.1	40
10 mμ.† ACTH¶	0				
	9	1.4	..	3.2	31
1000 mμ. ACTH	17				
	22	1.3	..	4.1	116

* Adrenal plasma flow was calculated from the adrenal blood flow and hematocrit.
** 17-OH corticoids is used for 17-hydroxycorticoids.
† mμ. = International milliunits.
‡ Lyophilized Upjohn Adrenocorticotropin Hormone.
¶ Lyophilized Armour Adrenocorticotrophin Hormone.

which 1 unit of ACTH administered to hemorrhaged, normal dogs who were maximally secreting glucocorticoids, stimulated only aldosterone secretion. However, 1 unit of ACTH administered to hemorrhaged hypophysectomized dogs always stimulated both glucocorticoid and aldosterone secretion.

Angiotensin II is the most potent vasopressor material known. Nephrectomy and hemorrhage increase the sensitivity of animals to angiotensin.[8] It is not surprising, therefore, that a dose as small as 3μg produced a pres-

sor effect. Although the present experiments do not rule out the possibility that the rise in blood pressure per se stimulated aldosterone secretion, several lines of evidence do not support this possibility. Laragh, *et al.*[10] have shown in man that the pressor agents norepinephrine, epinephrine, ephedrine, and vasopressin had variable effects on alosterone secretion except in sodium-depleted subjects with high aldosterone secretion in whom medullary type hormones generally produced a fall. Infusion of angiotensin II always produced a rise in aldosterone secretion. Moreover, Newman, *et al.*[15] demonstrated that norepinephrine does not increase aldosterone secretion in the dog, at least not in amounts which maintained the mean arterial pressure at 100 mm. Hg. Barter, *et al.*[1] actually dissociated alterations in blood pressure and aldosterone secretion in a specific experimental situation. They reported that bilateral constriction of the common carotid arteries in dogs with both thyrocarotid arterial junctions denervated, produced no increase in aldosterone secretion despite a rise in blood pressure due to an intact Hering reflex. Constriction, with the nerves to the thyrocarotid arterial junctions intact, resulted in increases in both aldosterone secretion and blood pressure. The work of Hilton, *et al.*[7] dissociated the cortisol stimulatory activity and the pressor action of several vasopressin peptides in the isolated perfused dog adrenal. Moreover, acetyl arginine (vasopressin) which has no pressor activity stimulated both cortisol and aldosterone in one experiment. These authors also stated that oxytocin and norepinephrine in doses sufficient to have pressor activity had no effect on cortisol secretion. Finally, in a study of the effect of hemorrhage on adrenocortical secretion in hypophysectomized dogs, the authors[13] found that transfusion of the shed blood did not cause a significant increase in adrenocortical secretions, including aldosterone, despite a modest elevation of blood pressure and increased adrenal blood flow. Nevertheless, these experimental situations do not duplicate the present experiments and it remains to be shown that the stimulatory effect on aldosterone secretion is specific for angiotensin.

It is tempting to speculate that angiotensin II is the elusive aldosterone-stimulating hormone. This hypothesis is supported by: (i) evidence[19] which suggests that the juxtoglomerular apparatus is a "volume" receptor; (ii) the hyperplasia of zona glomerulosa of the adrenal gland following renin injections;[4] (iii) the aldosterone-stimulating effect of angiotensin II in man[5, 10] and the dog;[8] and (iv) the increased secretions of aldosterone in man in malignant hypertension[11] and in some cases of hypertension due to unilateral renal vascular disease.[9] However, if angiotensin II is the aldosterone-stimulating hormone, a dose insufficient to have a pressor effect

should be able to stimulate aldosterone secretion, since several stimuli which increase aldosterone secretion are not associated with a pressor effect. Furthermore, a prolonged infusion of angiotensin II should maintain an increased secretory rate. Finally, and most important, it must be shown that the kidney and the renin-angiotensin system are necessary for the physiological stimuli which increase aldosterone secretion.

SUMMARY

The pattern of adrenocortical secretions produced by an infusion of angiotensin II was dose-dependent. A dose of approximately 30μg. of angiotensin II stimulated both glucocorticoid and aldosterone secretion. Reduction in dosage reduced the increment in glucocorticoid secretion without appreciably reducing the increment in aldosterone secretion. Angiotensin II always produced a pressor response.

The pattern of adrenocortical secretion produced by ACTH administration was also dose-dependent. Like angiotensin II, the larger doses of ACTH stimulated both glucocorticoid and aldosterone secretion. In contrast to angiotensin II, the smaller doses stimulated only glucocorticoid secretion. The possible role of the renin-angiotensin system in the regulation of aldosterone secretion is briefly discussed.

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MEDICINE AND SOCIETY IN AMERICA, 1660-1860. By Richard H. Shryock. New York, New York University Press, 1960. 182 pp. $4.00.

Dr. Shryock has written a book which is far and away the best general work on the early development of American medicine. His story covers the first two centuries of medical practice in what is now the United States. He terminates his history just before the onset of the Civil War, a political event immersed in medical history like most modern war. Dr. Shryock selected a "natural" terminus.

The author has not confined himself to depicting the practice of medicine alone but has shown how other events, social and scientific, here and abroad, participated in the developments which brought American medicine to the position it occupied a hundred years ago.

Four chapters constitute the book. Originally they were the Anson G. Phelps Lectures given at New York University in 1959, and since they average over 40 printed pages, the author must have expanded them. He has thoroughly documented his presentation with from 55 to 86 bibliographical notes following each chapter. These references by themselves make up perhaps the best bibliography of history of early American medicine available. It has the incomparable advantage of having been selected by the field's most outstanding scholar.

This "brief interpretation of medical developments during the first two centuries of American experience" is without peer. It is sophisticated medical history at its best.

F. G. K.

SURGERY IN WORLD WAR II. NEUROSURGERY VOLUME II. R. G. Spurling and B. Woodhall, Eds. Washington, D. C., Department of the Army, (Office of the Surgeon General), 1959, 705 pp., 283 illus. $7.00.

This contribution to the official history of the Medical Department of the U. S. Army in World War II is a fine addition to the clinical series of publications. Commendably edited by R. Glen Spurling and Barnes Woodhall, it is a volume containing a remarkably complete consideration of spinal cord and peripheral nerve injuries. The peace-time incidence of these conditions is sufficiently great to confirm their importance, but only such a comprehensive study as this can do full justice to the many varied aspects which merit attention. Each chapter is written by a recognized authority and is based upon extensive personal experience.

The first part of the book, devoted to spinal cord injuries, contains chapters upon the zone of interior and field problems, compound spinal cord injuries, urological aspects, general management of paraplegic patients, and ruptured intervertebral disks. Herein is recounted the splendid, cooperative achievement of 'all hands' in the management of paraplegia.

The second part covers the field and interior zone activities in relation to peripheral nerve injuries; methods of examination, anatomic aspects, surgical technique, and variously complicated injuries. There are excellent chapters on causalgia, nerve grafts, neuropathology, physical therapy, and

orthopedic techniques for use in irreparable nerve injuries. Under no other single cover can one find the diversity of information so authoritatively presented upon this subject.

WILLIAM J. GERMAN

INSULIN. F. G. Young, ed. British Medical Bulletin, Vol. 16, No. 3, London, The British Council, 1960. 87 pp. (175-264). $3.25.

This special number of the British Medical Bulletin has assembled 19 authors, all recognized experts, to discuss insulin in its various aspects. Dr. C. H. Best reviews the history of the discovery of insulin; F. Sanger describes the methods used in determining the amino acid sequence of the hormone; Randle, Taylor and Vallance-Owen discuss the problem of assaying insulin in blood; Fisher, Folley, Greenbaum, Korner and Manchester review the effects of the hormone on carbohydrate, fat and protein metabolism. A final section concerns clinical problems related to insulin. In addition, excellent papers on the amino acid composition of pituitary polypeptide hormones, by I. Harris, and the metabolism of peptide hormones by Kenny, although not entirely concerned with insulin itself, apply techniques learned in studying insulin to additional important problems.

None of the papers presents new material, but the reviews are easy to follow and offer an excellent introduction for the novice and refresher for the sophisticated.

PHILIP K. BONDY

THE THYROID GLAND. N. B. Myant, ed. British Medical Bulletin, Vol. 16, No. 2, London, The British Medical Council, 1960. 85 pp. (89-174). $3.25.

This issue, published in relation to the Fourth International Goitre Conference in London, 1960, includes articles by thyroidologists from England, Australia, and New Zealand. However, an international interest in thyroid disorders is prominent in these concise pages, with extensive bibliographies of between 40 to 100 references for each of the 15 articles.

Sir Charles Harington's introduction summarizes some striking developments of research on the thyroid gland during the last ten years. Emphasized are problems of transport of thyroid hormones in the blood, the "still vexed question whether Graves' disease is an autonomous disorder of the thyroid gland," and import of genetic factors on thyroid dysfunction.

Familial incidence of thyroid disorders could not be doubted after study of the explicit articles by Trotter and by McGirr on deafness and on sporadic goitrous cretinism. That genetic errors in synthesis of thyroid hormone frequently are partial and not complete is exemplified by McGirr's data on siblings in families with thyroid dysfunction. In physiological context is Eary's summary on "Central Nervous System and the Thyroid." From experiments, frequently on animals, he stresses that in spite of the reversibility of mental sequelae in adult thyroid underfunction, only prompt, early replacement therapy for hypothyroid young animals or persons can prevent irreversible mental sequelae. The occurrence of an abnormal thy-

roid stimulating hormone in patients with Graves' disease is postulated by Purves and Adams. A similar lack of dependence on TSH is described by I. Doniach during rapid growth of the infantile thyroid and the compensatory hypertrophy of thyroid remnants after thyroidectomy. Interrelations of TSH and ACTH are correlated by Brown-Grant. His observations include the effects on thyroid activity of cold temperatures and emotional stimuli in animals with or without pituitary stalk section.

Exogenous causes of goitre are described not only in Selwyn Taylor's paper on "Genesis of the Thyroid Nodule" and iodine lack, but also in Clements' article "Naturally Occurring Goitrogens." Writing from the Institute of Child Health in Sydney, Clements gives on the spot information about goitrogens in milk, pasture, and crops of Australia.

Investigators who enjoy Dr. Pitt-Rivers' publications might consider her article on "Biosynthesis of Thyroid Hormones" as an appetizer rather than a *pièce de resistance.* Data in four pages on "Transport of Thyroid Hormones" have condensed much pertinent information. Nevertheless no four pages could compete with the comprehensive articles on thyroxine-binding-proteins published during the last year by Ingbar, Robbins, and Rall.

Two independent papers attract serious attention: Golden's summary of various diagnostic tests with radioactive iodine might well be required reading for clinicians who rely on I^{131} without choice of the precise test appropriate to the diagnostic problem. That I^{132} is a safer medium than I^{131} is clearly and definitely stated. In five pages Dr. Roitt and Dr. Deborah Doniach discuss thyroid auto-immunity tests with pertinent references to an extensive bibliography. Hashimoto's disease has been diagnostically differentiated from thyroid carcinoma by measurement of thyroid auto-antibody titres. Patients with exophthalmos had high titres in their sera.

As a source book this issue of the British Medical Bulletin, with inclusion of extensive bibliographies, is an invaluable reference book on interrelating functions of the thyroid with physiological processes and with activity of other target organs under control of the anterior pituitary.

EVELYN B. MAN

LIPIDE METABOLISM. Konrad Block, ed. John Wiley and Sons, Inc., New York, 1960. 411 pages. $10.50.

This excellent book reviews developments in the field of lipide metabolism focusing on those occurring between 1955 and 1959 or early 1960. Older work is cited where it contributes to the historical development of each subject. The book does not pretend to be comprehensive, but all major recent developments are thoroughly, lucidly, and briefly covered. Careful reference to other reviews is given for the older literature or for subjects not covered in detail in the present treatise.

The book is divided into eight chapters, each written by experts in the

topics under discussion. The scope of the book is indicated by the titles of the chapters: Enzymatic mechanisms of fatty acid oxidation and synthesis; The metabolism of the unsaturated fatty acids; The metabolism and function of phosphatides; Metabolism of glycerides; Lipolytic enzymes; Hormonal regulation of fatty acid metabolism; Formation and metabolism of bile acids; Chemistry and metabolism of bacterial lipides. Each chapter is concluded by a long list of references.

The orientation of the book is physiological and biochemical rather than clinical although reference is made to disease, human or animal, where it is pertinent. Each author has not merely catalogued information but has attempted to assemble a huge body of diversified data into the hypothesis most consistent with the known facts at the time of publication.

This book will be most helpful to everyone working in the field of lipid metabolism.

MARGARET J. ALBRINK

PRACTICAL CLINICAL MANAGEMENT OF ELECTROLYTE DISORDERS. By William J. Grace. New York, Appleton-Century-Crofts, Inc., 1960. 132 pp. $4.95.

This small volume is intended, as the title indicates, to give attention to problems of electrolyte derangement from the clinician's point of view. It is a brief guide to many of the important chemical imbalances resulting from organic dysfunctions, such as cardiac failure, renal failure and diabetic acidosis. However, the book does not have the format of a handbook of therapy. It presents illustrative case histories, including the therapy actually administered, and discusses each group of cases briefly, indicating the theoretical basis, and in some instances the weaknesses, of the treatment. There is then a capsule discussion of the treatment the author advocates, often (properly) dogmatic as in the discussion of control of diabetic acidosis. Throughout, the style is fluent and the exposition clear; in all, the book is a painless rendering of the subject for someone who has gone through the more physiologically-oriented larger texts. One wonders, in this regard, whether this book really fills any great gap in a field with so many commendable works in it. Although clinical problems are not given central consideration in these larger works, they are, nevertheless, competently presented and discussed in most of them. Judging this book on its merits alone, it is certainly a quick, accurate survey of the clinical side of electrolyte problems. There are one or two disturbing points, such as the placing of calcium and digitalis in the *denominator* of a ratio of cardiac irritability, despite an accurate accompanying discussion of their effects, (a typographical error?) and calling generalized amino-aciduria "cystinuria" rather than "cystinosis," but there are no glaring errors or gross oversimplifications, the latter particularly admirable in a brief work. This book, then, is a fine work for second year medical students eager for a clinical approach to the subject, and for junior house officers several

years removed from their initial contact with the subject in more extensive monographs.

M. S. M.

PHOSPHOROUS METABOLISM OF BRAIN. By P. J. Heald. New York, Pergamon Press 1960, 195 pp. $6.50.

This small book amply fulfills the promise of its title; it is one of the best books on a specific aspect of brain metabolism to appear in recent years.

The first two chapters cover phosphorus metabolism in the brain *in vivo* and include studies on the levels of phosphorylated compounds in the brain under various conditions. In addition, changes in the phosphate levels in the developing brain are also considered. The next three chapters are concerned with experiments *in vitro* on phosphate metabolism. These discussions are followed by a chapter in which the author attempts to integrate our knowledge on phosphate metabolism. The final chapter consists of an appendix listing the recommended techniques for studying phosphate derivatives in cerebral tissues.

Not merely a compilation of data on this subject, the author constantly and critically assesses the experimental findings with regard to the techniques that were employed. In addition, Dr. Heald does not fail to see the forest for the trees and continually attempts to relate function to metabolism. That this marriage is rarely successful is not due to an inadequacy of the author but reflects primarily the deficiency of our knowledge of cerebral events. Finally, for the novitiate in this field, the detailed descriptions on the preparation of tissues and determination of phosphorylated compounds will be of immense assistance.

One error noted in the author index was the assignment of references for T. M. Brody to B. B. Brodie.

JACK R. COOPER

LECTURES ON HEMATOLOGY. F. G. L. Hayhoe, ed. New York, Cambridge University Press, 1960. 247 pp. $11.50.

As medical science gets more complex, review papers by authorities in specialized fields of investigation become progressively more important to maintain scientific communication. This book, *Lectures on Hematology*, is a compilation of thirteen lectures delivered at Cambridge, December 1959, as a post-graduate course; the thirteen lecturers are indeed outstanding investigators in certain fields of hematology, and their papers present comprehensive reviews of their own areas of research interest. Hematology is by no means broadly covered, but the lectures generally fall into three fields: red cell problems, leukemia, and coagulation.

In the red cell field, Dr. Dacie discusses auto-immune hemolytic disease extremely well, and his comments ably serve as general guides for therapy of this process. Dr. Davidson covers the field of iron metabolism in a comprehensive and rather novel manner, going from phylogenetic considera-

tions and basic biochemistry of iron to practical iron therapy. His writing style and word usage, combined with his immense knowledge, make this a particularly outstanding lecture. In reviewing the megaloblastic anemias, Dr. Girdwood draws extensively on his own experimental observations. His simple classification of megaloblastic anemias into those related to deficiency of folic acid and those due to deficiency of vitamin B-12 is most useful for clarifying our thought processes; from there on, discussion of the pathogenesis of the deficiencies makes good sense and mixed avitaminoses are logically expected and found. Dr. Discombe's lecture on transfusion hazards was rather disappointing. In his introduction he indicated the need for a comprehensive and scientific appraisal of all the transfusion risks, but then he fell far short of supplying that need and his lecture tends towards a series of personal anecdotes illustrating various transfusion malpractices. Dr. J. C. White discusses abnormal hemoglobins, emphasizing particularly their chemical characteristics, but tying these characteristics to a brief clinical and genetic discussion.

The leukemia section is introduced by the chief editor of the volume, Dr. Hayhoe, who provides a sound article on the classification of leukemias based on clinical and cytological features of the diseases. Dr. Hayhoe's application of a variety of special staining techniques to differentiation of the cells in acute leukemia undoubtedly provides a refinement in cytologic criteria.

Following Dr. Hayhoe's paper on classification, Dr. Wetherley-Mein provides a rather brief and rather general discussion of the interrelationships of diseases of the hematopoietic system as 'The Myeloproliferative Syndromes.'

Dr. Richard Doll's review of perplexing problems raised by statistics of leukemia incidence is indeed stimulating. He points out the several types of leukemia seen in certain countries, particularly the paucity of chronic lymphatic leukemia in Asians. He emphasizes both the changing pattern of statistics of acute leukemia in children, and the wide variation in incidence of the diseases relative to occupation. All of this points to important environmental factors in the etiology of leukemia.

Treatment of leukemia is covered well by Dr. Galton, who stresses the basic difference between chronic lymphatic leukemia and chronic granulocytic leukemia, and how these differences influence therapeutic considerations. One relatively new chemotherapeutic development that Dr. Galton brings into focus is the value of prolonged adrenal steroid therapy in advanced chronic lymphatic leukemia. Another therapy, marrow transplantation after total body irradiation, is discussed extremely well by Dr. Kay. The latter reviews the animal studies done on this problem and discusses their relationship to transplantation in man, concluding that such an approach has little chance of success as a practical therapy.

In the final three lectures on hemostasis, Dr. Hardisty opens with an extraordinarily complete review of all the factors that control hemostasis—the coagulation factors and the vascular ones. In the process, he provides an excellent scheme of coagulation factors as currently recognized. Dr. Ingram covers the coagulation defects more specifically, in a clinically and

therapeutically oriented manner. Dr. Ackroyd completes the volume by a review of purpura, covering both vascular defects and thrombocytopenia and reviewing his own outstanding studies of the pathogenesis and abnormal physiology of drug-induced thrombocytopenia.

J. W. HOLLINGSWORTH

THE FUTURE OF MAN. Predictions of a Biologist. By P. B. Medawar. New York, Basic Books Inc., 1960, 128 pp. $3.00.

This book proves that scientific distinction and the ability to write lucidly and entertainingly are not necessarily incompatible. Many fascinating points are raised, few definitive answers are given. Why do children born between May and October have slightly higher scores in intelligence tests than children born between November and April? Is human intelligence declining? Can population be stabilized? These and many other questions are discussed in a most readable fashion.

HENRY G. MAUTNER

COLD INJURY. Steven M. Horvath, ed. New York, Josiah Macy, Jr. Foundation, 1960. 375 pp. $6.50.

The Josiah Macy Jr. Foundation has published the 1958 Conference on Cold Injury. The title is misleading in that no pathologic state is described but rather they have discussed the current knowledge of physiologic adjustments to a cold environment. Much of the material is of a basic science nature and the publication is in dialogue form. Although this is a cumbersome method it proves useful in underscoring certain points and in suggesting other interpretations of the evidence presented.

In the early chapters, the mechanism of thermal regulation in experimental animals is discussed and it is interesting to note how the production of specific central nervous system lesions has localized control centers. Following, is a discussion on hibernation—a process that remains poorly understood at this time but would be of tremendous value if it could be reproduced in man.

In contrast to these presentations were the chapters on living conditions at the South Pole in which were described the hardships endured by one hundred men at temperatures as low as -40°F for 6 months or more. Many practical points were discussed, for example clothing requirements, caloric needs, psychologic adjustment, and work productivity of the men under these trying circumstances.

The highly technical level of discussion is resumed in the presentations on temperature gradients in fluid systems, measurement of wind chill, and physiologic heat production. The final chapters concern experiments on human subjects. In one, the volunteer was placed in a cold ward at *15.6°* C. and energy balance studies were undertaken. In the last chapter are presented field studies among the Australian aborigines and their adaptation to a cold environment.

This book has achieved a degree of balance between the theoretical and the practical. It is far from a didactic exposition on man's adjustment to

cold but rather its purpose is to define the perimeter of our knowledge and areas for future study.

GEORGE F. GOWEN

EPIDEMIOLOGIC METHODS. By Brian MacMahon, Thomas F. Pugh and Johannes Ipsen. Boston, Little, Brown and Company, 1960. 290 pp. $7.50.

During the past several years there has been a growing interest in epidemiologic investigation, particularly in the field of non-infectious diseases. Although a few books devoted to epidemiology are available, for the most part the subject has been presented in public health and preventive medicine textbooks, in which methodology has often taken second place to a descriptive epidemiology of specific diseases. The present volume considers the subject as a science and, as suggested by the title, it is constructed on a basic framework of epidemiologic methodology. Major emphasis is placed on concepts and principles, most of which are well established in the field of infectious disease epidemiology, and their modification and application for use in present day studies of non-infectious diseases.

The epidemiologic concepts of direct and indirect causal association and various concepts of classification and grouping of illnesses are well presented in abstract and diagramatic forms followed by examples drawn from established epidemiologic experience. The important areas of terminology, methods of measurement and data collection are clearly presented with, as far as possible, an intentional avoidance of purely biostatistical techniques. In fact, in the preface it is suggested that statistical knowledge is not required in order to understand the principles of epidemiology, but "the reader intending to put these principles into practice would do well to sentence himself to a term with a book of elementary statistics."

The major portion of the book is concerned with various axes of classification (e.g. time, place and person) and their sub-divisions most commonly used in analyzing associations between characteristics of the population under study and its entire environment, temporal and spatial, in relation to disease patterns. Instead of describing the total epidemiologic picture of individual illnesses, chapters or sections are devoted to specific characteristics and for each of these several examples have been drawn from a wide variety of diseases.

Two clearly written chapters on analytic epidemiology (cohort studies and case history studies) will prove of considerable interest and value to all who have experienced difficulty in understanding the problems and limitations of such studies and have had doubts about their proper interpretation in view of conflicting testimonials (e.g. lung cancer and cigarette smoking).

The final chapter entitled "Experimental Epidemiology" is limited to human experiments, controlled, uncontrolled and "natural." Only fleeting mention is made (in an earlier chapter) of the "old" experimental epidemiology of Greenwood, Topley and Wilson. One wonders why this field, animal as well as human, failed to receive more attention in a book which provides such complete coverage of the methods of epidemiologic

investigation. Also lacking are discussions of serologic epidemiology (antibody and biochemical) and the associations between blood groups and various diseases.

In spite of these relatively minor shortcomings, this book fills a definite need. It will prove to be valuable to a wide variety of students and investigators in the medical field in addition to those primarily concerned with epidemiology.

ROBERT W. MC COLLUM, JR.

BIOCHEMICAL PREPARATIONS, VOL. 7. Henry A. Lardy, ed.-in-chief. New York, John Wiley & Sons, Inc., 1960. 102 pp. $5.25.

Previous volumes of "Biochemical Preparations" have been received gratefully by the experimental biochemist, as a rule. The current volume should be a welcome addition to the six which have appeared at 1-3 year intervals since 1949. It is in the nature of such a publication, of course, that it cannot possibly meet all specific demands. The only criterion one might use in deciding on the degree of general usefulness of a publication entitled "Biochemical Preparations" is the diversity of selection. This requirement has been met well in past volumes and the current one is no exception. Volume 7 contains twenty preparations, assembled by an editorial board headed by H. A. Lardy. Each preparation had been checked independently before inclusion in the volume. This procedure, also used in the past, should insure reliability and reproducibility. Most of the preparations described in this volume deal with substances not previously included in "Biochemical Preparations"; among them are N-acetylneuraminic acid, β-aminoisobutyric acid, carbamyl phosphate, carnitine chloride, cerebrosides, coproporphyrin III tetramethyl ester, 6-deoxy-6-fluoro-D-glucose, dihydroxyacetone phosphate, flavin adenine dinucleotide, D-fructose-1-phosphate, 3-hydroxy-L-kynurenine, hydroxypyruvic acid phosphate, methylmalonic semialdehyde, stearic acid, stigmasterol, and tetrahydrofolic acid. In addition a new procedure for the preparation of the acid potassium salt of isocitrate is included to supplement a previously described preparation of isocitric acid as its lactone or ester derivative. A long-chain fatty acyl coenzyme A preparation (palmityl CoA) supplements the prior description of the preparation of succinyl CoA. Finally two sections deal with the enzymatic and chemical synthesis of P^{32}-labeled adenosine phosphates. In one respect, the current volume differs from previous ones significantly. For the first time, not a single enzyme or other protein preparation is included. This is perhaps regrettable and it is hoped that this is not a result of a permanent change in editorial policy. The organization of each procedure follows the format or prior years. A statement of the principle of the preparation is followed by a description of starting materials and the detailed procedure. Comments made by the checkers are recorded in footnotes. Each section is concluded by a description of the properties and the purity of the material and by a list of references to other methods of preparation. A statement, related to the best conditions of storage and the stability of the substance in question, would be a very useful addition if it were to be made required information in the future. Such

information is not always included in the current volume or in past years. In any case, this latest effort of the editors, contributors and checkers of "Biochemical Preparations" will be appreciated by experimental biochemists.

GEORGE TABORSKY

A Syllabus of Laboratory Examinations in Clinical Diagnosis. (Successor to Thomas Hale Ham's Syllabus) Critical evaluation of laboratory procedures in the study of the patient. Lot B. Page and Perry J. Culver, eds. Cambridge, Massachusetts, Harvard University Press, 1960. 580 pp. $12.50.

Physicians, particularly internists, pediatricians, and house officers, have traditionally performed simple laboratory tests on their patients. A great spurt in medical education occurred in the past century when the young physician was introduced into the clinical laboratory to obtain data on his patients by means beyond that of a history and physical examination. A few physicians of the old school still retain respect for examination of a peripheral smear, the urine sediment, or the test for occult blood, and perform these tests themselves as an integral part of their examination of the patient. The younger, hurried physician or student may want to know the plasma cortisol before he has inspected the peripheral smear or the endogenous creatinine clearance before he has seen the urinary sediment. He is not acquiring the direct laboratory experience that his predecessors had. Following this book he can easily do so.

The Syllabus of Laboratory Examinations started by Ham and continued by Page, Culver, thirty-one contributors and sixteen critics (mostly from the Harvard family) provides detailed technical information which enables physicians and particularly house officers to perform tests. Explanations are provided for physiologic variations and pathologic physiology. The chapters cover definition of units, range of normal values for an extensive list of tests, statistics, techniques of hematology, elements of blood banking, urine examination, interpretation of various organ functions, endocrinology, cardiac catheterization, and many other significant topics.

The Editors are to be commended for having integrated the efforts of many collaborators. That portability has been sacrificed is apparent from the size of the volume: 580 pages and 1440 grams. It is not to be carried in the side pocket! Nevertheless, the volume is easy to read, easier than the previous edition, and it has a hard cover compared to the previous soft one.

The Syllabus is highly recommended for inclusion on the bookshelves of practicing physicians, medical investigators, house officers, students, and technicians as a valuable reference for information on how to do a test or interpret it in a field currently called Clinical Pathology. It is the best of its kind today.

DAVID SELIGSON

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Lucké, Baldwin and Rea, M. H.: Studies on aneurysm. *J. Amer. med. Ass.*, 1921, *77*, 935-940.

Barcroft, Joseph: *The respiratory function of the blood.* Cambridge, Cambridge University Press, 1914, pp. 58-61.

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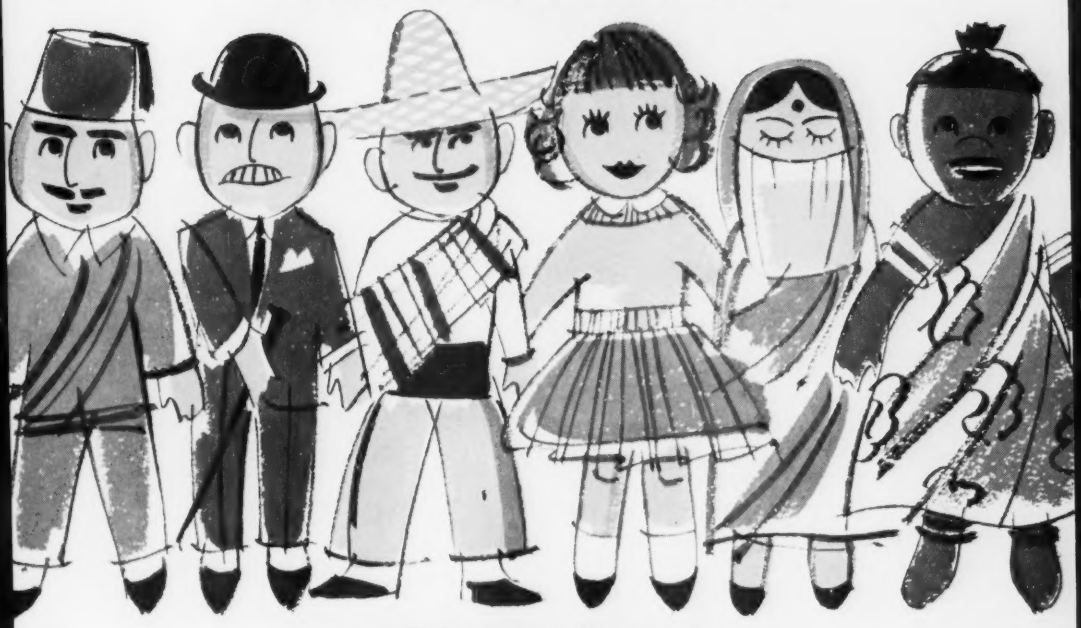

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